STUDIES ON THYROIDAL PROTEIN BIOSYNTHESIS IN RELATION TO THYROID HORMONE BIOSYNTHESIS

by

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I hereby declare that this thesis and the experiments described therein, unless otherwise indicated, are my own work.

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SUMMARY

1. The object of the study was the isolation from thyroglobulin of peptides containing iodothyronines or iodotyrosines, followed by investigation of these peptides as the actual or potential sites of thyroid hormone synthesis in thyroglobulin.

2. A cell free preparation capable of incorporating amino acids into protein was isolated from rat thyroid. Some of the unusual properties of this preparation were studied.

3. Thyroglobulin containing ^{14}C-amino acids was isolated from whole rat thyroids after incubation with ^{14}C-amino acids. Under similar conditions sheep thyroid slices rapidly incorporated ^{14}C-amino acids and $^{131}I^-$ yielding thyroglobulin highly labelled with both isotopes.

4. Thyroglobulin isolated from sheep thyroid slices was purified, by fractionation with ammonium sulphate or by gel filtration on Sephadex G-200. The purity of the preparation was determined by a variety of methods including gel filtration, electrophoresis and ultracentrifugation. Sucrose gradient centrifugation revealed a highly iodinated lighter protein fraction.

5. Labelled thyroglobulin was hydrolysed by α-chymotrypsin and the resulting hydrolysate subjected to electrophoresis followed by chromatography at right angles (peptide mapping). Autoradiograms of these peptide maps revealed the presence of a number of peptides labelled with $^{131}I_2$ and a larger number labelled with ^{14}C-tyrosine. None of the ^{131}I-peptides was unequivocally labelled with ^{14}C-tyrosine.

6. The most highly labelled ^{131}I-peptides were isolated, in some cases purified by electrophoresis at pH 8.2 , and their iodoamino acid contents found after pronase hydrolysis and chromatography. Each peptide contained only one iodotyrosine confirming the specific nature of tyrosyl iodination indicated by the small number of intensely labelled ^{131}I-peptides compared with a larger number of ^{14}C-tyrosine peptides.

7. The approximate sizes of the ^{131}I-peptides were determined by estimation of the bonds hydrolysed by α-chymotrypsin and by gel filtration of the peptides on Sephadex G-25.

8. During the incorporation of $^{131}I^-$ into thyroid slices over a period of 5 hr. the activity of each ^{131}I-peptide, as a percentage of the total $^{131}I_2$ in thyroglobulin, did not alter although the ratio of the ^{131}I-activities of mono- to diiodotyrosine fell throughout this period.

9. Monoiodotyrosyl peptide A_5 was iodinated chemically with $^{131}I_2$. The product was not identical with already isolated diiodotyrosyl peptides of similar mobilities on peptide mapping.

10. Peptide A_5 was associated with another peptide (separated by electrophoresis at pH 8.2) whose products of iodination were identical with those of A_5 and which, it is suggested, is the uniodinated form of A_5.

11. This latter peptide taken together with A_5, is present as 4 moles per mole of thyroglobulin as determined by iodination with $^{131}I_2$ of known specific activity. Two other peptides, N_9 and N_{9a}, are each present as 5 moles per mole of thyroglobulin. It is suggested that this confirms the evidence that thyroglobulin comprises four identical sub-units and that iodotyrosyl residues can remain partially uniodinated as well as being mono- or di-iodinated.

GENERAL INTRODUCTION

Of the several stages in the biosynthesis of the iodothyronines* perhaps less is known about the last stage than any other. This stage involves the coupling of a mono- or diiodotyrosyl residue with a diiodotyrosyl residue in thyroglobulin, which results in the formation of an iodothyronyl residue. The coupling reaction might possibly be mediated by a 'coupling enzyme', or by a specificity in the structure of thyroglobulin enabling or encouraging the transfer of an iodinated phenolic group from one iodotyrosyl to another. Isolation and characterisation of the site of iodothyronine synthesis will reveal something of the coupling mechanism.

The information used to trace the progress of our understanding of iodothyronine synthesis comes from two phases of research. The earlier studies dealt mainly with isolation of iodinated compounds from thyroid, and with thyroidal iodine metabolism. More recently, as the techniques of protein chemistry developed, a great deal of interest has been shown in the properties of the various iodinated proteins from the thyroid. Further elucidation of the last stage of iodothyronine synthesis is intimately bound to investigation of iodoprotein synthesis and this work is intended to connect our understanding of iodine metabolism and protein synthesis in the thyroid.

Survey of iodine metabolism in thyroid to date

Although early work on the incidence of endemic goitre (for a later review of this material see Orr and Leitch, 1929) had indicated

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* The thyroid hormones are considered to be thyroxine and 3,5,3′-triiodothyronine, and will be referred to as such, separately, and as the iodothyronines collectively.

a relationship between iodine intake and goitre, the hormonal form of the iodine was not isolated until 1915 when Kendall isolated thyroxine from a sodium hydroxide hydrolysate of pig thyroid. Kendall's proposed structure for thyroxine, based on an indole, was shown to be incorrect by Harington (1926,a). The latter, using a baryta hydrolysis based on the method of Oswald (1909), achieved much better yields of thyroxine and he correctly determined the structure of the iodothyronine moiety. Orientation of the iodine atoms was established in collaboration with Barger (Harington and Barger, 1927).

The following year Harington and Randall (1929), using peptic and tryptic digestion, as well as the baryta hydrolysis, isolated diiodotyrosine from desiccated thyroid. Harington, after considering the amounts of iodoamino acids recovered and the quantity of iodine produced by their destruction during hydrolysis, concluded that diiodotyrosine and thyroxine were the only iodine-containing compounds in the thyroid.

When $^{131}I^-$ became available Fink and Fink (1948) showed that one to two days after injection of $^{131}I^-$ into a rat, baryta hydrolysis of the thyroid revealed, after chromatography and autoradiography, not only iodide, thyroxine and diiodotyrosine, but also a spot identified as monoiodotyrosine.

Gross and Pitt-Rivers (1952), also using the technique of *in vivo* iodination of rat thyroid with $^{131}I^-$, were able to demonstrate a compound shown to be identical with 3,5,3′-triiodothyronine. Roche, Lissitsky and Michel (1952a, b), at the same time and using the same technique, confirmed this result and chemically synthesized 3,5,3′-triiodothyronine from 3,5-diiodotyrosine.

Three of the main concepts of iodine metabolism which are now accepted virtually without reservation, namely the ability of the

thyroid to concentrate iodide from the surrounding medium, the coupling of two diiodotyrosines to give thyroxine, and the iodination of tyrosine, were put forward at quite an early stage. Firstly, Blum and Grützner (1914) administered potassium iodide to a dog and detected an increased level of iodine in its thyroid. This result was confirmed by Marine and Rogoff (1916) who found that both *in vivo* and during *in vitro* perfusion, dog thyroids took up, and largely retained, administered iodide. The second step came when Harington (1926,b) suggested that two diiodotyrosines reacted to form thyroxine. Lastly, Blum (1927) put forward the idea that the thyroid contained an 'iodase' which would oxidise iodide to iodine allowing the iodination of tyrosine.

As thyroglobulin is the source of iodothyronines in the thyroid gland, *in vitro* iodination of this protein, and others, was carried out (Roche, Michel and Lafon, 1947, and Roche and Michel, 1951) in an attempt to discover any property of the iodination or of the iodinated proteins which was unique to thyroglobulin and might provide an explanation for the *in vivo* mechanism of iodothyronine synthesis. Chemical iodination of casein, zein and hog thyroglobulin under alkaline conditions showed that the latter yielded the least thyroxine, expressed as moles of thyroxine synthesized per mole of initial tyrosine. For a given number of tyrosyl residues reacting with iodine those in casein are iodinated to a greater extent and the yield of thyroxine is higher. Nevertheless both casein and thyroglobulin give rise to thyroxine in quantities corresponding closely to their total tyrosine contents. Thyroglobulin, which already has its physiological content of iodine and iodoamino acids, has no greater aptitude for the further formation of thyroxine artificially.

Only after the iodination of the above proteins had resulted in a large increase in the diiodotyrosine content was there any synthesis of thyroxine. This was in contrast to the *in vitro* iodination of silk fibroin (Roche and Michel, 1951) where a considerable content of diiodotyrosine was accompanied by a very small quantity of thyroxine. X-ray analysis of the iodinated material showed that the iodinated residues were rigidly held far apart from each other.

Evidently, for the production of thyroxine, not only must the degree of iodination be sufficient, but it is probable that the tyrosyl residues must be sufficiently close, or in the correct orientation, for coupling to take place.

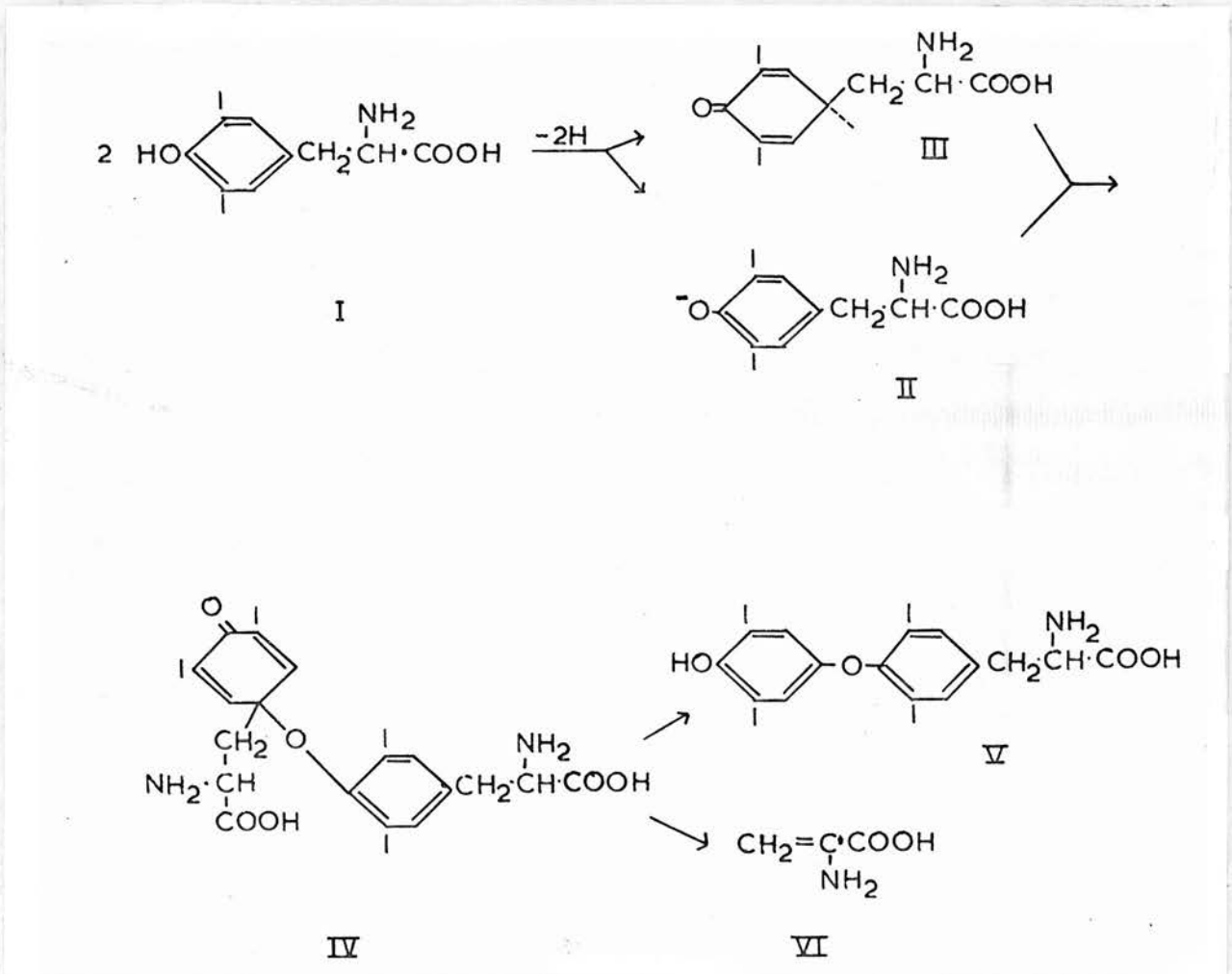
A later study on the *in vitro* iodination of hog thyroglobulin by Edelhoch (1962) showed that up to a level of 1.4% of iodine the protein structure was virtually unaffected, but above this level structural changes occurred. For each increment in iodination more thyroxine is formed until a stage is reached where further iodination produces only smaller and smaller increases of thyroxine (Roche and Michel, 1951). At this level of iodination, the ratio of the iodine in thyroxine to that in diiodotyrosine is still in the mammalian physiological range of 0.30 to 0.34 (Wolff and Chaikoff, 1944). Further iodination, causing a progressive degree of denaturation, leads again to increments in thyroxine content which pass through a maximum as before. The biphasic nature of this ratio points to there being a maximum number of thyroxyl residues produced physiologically (in sheep 3 moles/mole thyroglobulin). Chemical iodination to increase this number denatures the protein, making more residues accessible for iodination (Li, 1945).

Ludwig and Mutzenbecher (1939) iodinated casein in mildly alkaline buffer to yield, after hydrolysis, thyroxine as well as mono- and diiodotyrosine. In the same year the latter demonstrated the coupling *in vitro* of free diiodotyrosine at 37^{0} and pH 10 yielding small quantities of thyroxine.

Under similar conditions Harington and Pitt-Rivers (1945) showed that the reaction did not proceed in the absence of oxygen or in the presence of thiosulphate, and was accelerated by oxidising agents such as iodine or peroxide. The yield of thyroxine, calculated as the loss of diiodotyrosine, was 4%. Protection of the α-amino and α-carboxyl group increased yields. Pitt-Rivers (1948), using N-acetyl diiodotyrosine and N-acetyl diiodotyrosyl glutamic acid, found 25% and 35% conversion to the thyronyl peptide, respectively. The nature of the side-chain split off was not discovered. Sela and Sarid (1956) found that the quantity of serine released during the iodination of poly-L-tyrosine had no quantitative relationship with the production of thyroxine. During aerobic incubation of α-N-acetyl-ε-N-(N-acetyl diiodotyrosyl) lysine (Pitt-Rivers and James, 1958), α-N-acetyl-ε-N-(N-acetyl thyroxyl) lysine is formed in yields as high as 50% with molecularly equivalent amounts of ε-N-hydroxy-pyruvoyl-α-N-acetyl lysine.

The hypothetical mechanism for coupling proposed by Johnson and Tewkesbury (1942) although based almost entirely on *in vitro* model systems remains as satisfactory as any. Under conditions which encourage dissociation of the phenolic hydroxyl group of diiodotyrosine (I), such as alkaline conditions *in vitro* or perhaps electrostatic attractions *in vivo*, the negatively-charged molecule (II) may tautomerise to the free radical quinone form (III). The

latter is then attacked at the 1-position by the negatively-charged II resulting in the hypothetical quinolether intermediate (IV). Rearrangement and stabilisation leading to the formation of thyroxine (V) appears to occur by the expulsion of iminopyruvic acid (VI). Johnson and Tewkesbury (1942) claimed to have detected pyruvic acid and ammonia which would be the hydrolysis products of iminopyruvic acid.



Although the above scheme is offered only as a chemical speculation on the nature of the coupling reaction, it is interesting that a complex transfer reaction of this type can occur non-enzymically under mild conditions. The diiodination of tyrosyl residues in thyroglobulin has also been demonstrated in an artificial system by

De Groot and Davis (1961,a) who added hydrogen peroxide and $^{131}I^-$ to a thyroidal microsomal preparation containing peroxidase. These earlier experiments were concerned mainly with the isolation of iodinated compounds from the thyroid and also the in vitro mechanisms of coupling of iodotyrosines, which may give some insight into the in vivo methods of thyroxine synthesis.

Early in vivo work utilising $^{131}I^-$ in studies of iodine metabolism in thyroid did not quantitatively reflect the in vivo state because the large doses of carrier $^{127}I^-$ injected along with $^{131}I^-$ caused changes in metabolism. Perlman, Chaikoff and Morton (1941) showed, in both sheep and rats, that $^{131}I^-$ uptake into diiodotyrosine exceeded that of $^{131}I^-$ into thyroxine at all times, but that the latter fraction increased steadily with time. Mann, Leblond and Warren (1942) analysed dog thyroid for $^{130}I_2$ and $^{127}I_2$ at various times after the injection of $^{130}I^-$ to confirm the result of Perlman et al. above. Both groups concluded that diiodotyrosine was a precursor of thyroxine. Using rat thyroid slices Morton and Chaikoff (1943) found formation of diiodotyrosine and thyroxine from iodide in the buffer. Since 1948 monoiodotyrosine and triiodothyronine have been discovered and included in the scheme proposed by Pitt-Rivers and Tata (1959) for the synthesis of iodothyronines. This scheme comprises (a) concentration of iodide from circulation by thyroid, (b) oxidation of iodide to iodine with incorporation into tyrosyl residues yielding, firstly, monoiodotyrosyl and secondly, in some cases, diiodotyrosyl residues, (c) coupling of two diiodotyrosyl residues with the production of thyroxine, (d) synthesis of triiodothyronine either by the coupling of monoiodotyrosyl and diiodotyrosyl residues, or by the deiodination of already formed

thyroxine. This latter point will be discussed later.

Each of the above stages may be enzymically controlled and a large body of work has concentrated on the isolation and characterisation of enzyme systems from thyroid. Isolation of enzymes postulated by the above scheme would provide useful confirmatory evidence for its validity.

There are probably several peroxidases in the thyroid, not all of which catalyse iodination (De Groot and Davis, 1962, and Yip, 1965), but Tong, Taurog and Chaikoff (1957), using a mixed microsomal-mitochondrial preparation, demonstrated the production of iodotyrosines from protein bound tyrosyl residues. The <u>in vivo</u> source of H_2O_2 remains unknown. In an artificial system used by several workers to iodinate thyroglobulin to higher than normal levels H_2O_2 is generated by the oxidation of glucose with glucose oxidase and, in the presence of horse radish peroxidase, iodide is oxidised to iodine.

The gland can concentrate iodide to some 250 times its level in plasma under conditions of iodide lack, hyperplasia and goitre. This effect is inhibited by monovalent ions such as perchlorate, which are similar in size to iodide and may compete for transport across the cellular membrane. Iodide concentration requires energy as judged by the inhibitory effects of dinitrophenol and cyanide ion (Freinkel and Ingbar, 1955). Both an ouabain-sensitive ATPase and specific phospholipids have been suggested as a basis for the active iodide transport, but their roles remain doubtful.

Thyroglobulin must be hydrolysed before the iodothyronines are released into circulation. Robertis (1941) first showed by histochemical methods the presence of thyroidal protease in the colloid of single follicles of rat thyroid. Of the many protease preparations only the peptidase of Weiss (1953) had activity in the

physiological pH range; the remainder had pH optima in the range pH 2-4. However, as some or all of the proteolytic activity is associated with the lysosomes, and as these contain other enzymes, notably phosphatases and esterases, it is possible that they have a local pH lower than the general intracellular one.

Deiodinases in thyroid attack free iodotyrosines but not the iodotyrosyl residues in thyroglobulin (Roche, Michel, Michel, Gorbman and Lissitsky, 1953). The deiodinases are present in the particulate fraction of thyroid and, although Roche et al. (1953) found they would not deiodinate the iodothyronines, Tata (1958) found that removal of blood proteins from thyroxine allowed deiodination. It is possible that thyroxine is protected by being specifically adsorbed on to both thyroidal and body cell proteins in a way analogous to its binding to the prealbumin fraction in blood. In vivo, if the diiodotyrosyl residues are to be protected from enzymic deiodination sufficiently to allow coupling to occur, they must either be spatially separated from the enzyme within the cell, or, what is perhaps equivalent, bound in thyroglobulin.

Absences of the enzymes would be expected to present patients with familial goitre with hypothyroidism. Four or five types of hypothyroidism have been tentatively diagnosed as enzyme defects.

A defect in the iodide accumulation system has been well characterised by Stanbury (1960). Only a small fraction of administered radioiodine accumulated in the thyroid and slices from a biopsy specimen failed to concentrate iodide from medium. Administration of therapeutic doses of $^{127}I^-$ enabled enough iodide to diffuse into the gland allowing iodothyronine synthesis and the return of the patient to euthyroid state. The iodide transport system was also missing from gastric mucosa and salivary glands.

Iodide organification defect has been studied in several laboratories. Stanbury and Hedge (1950) showed that the thyroids of three sibling cretins rapidly took up $^{131}I^-$ from a dose of 100μC, reaching a plateau in 2 hr. After 27 hr. the administration of thiocyanate, to allow the efflux of free iodide from the gland, caused the rapid loss of 70 to 90% of the accumulated radioiodine. A later <u>in vitro</u> study with subcellular fractions from a similar gland showed this had no ability to iodinate tyrosine in contrast to preparations from normal human thyroids (Haddad and Sidbury, 1959).

Stanbury, Chela and Pitt-Rivers (1955) examined two sisters presenting goitre and hypothyroidism for a possible defect in iodotyrosyl coupling. Their uptake of radioiodine was extremely rapid and reached nearly 100%. Propylthiouracil, given to block organification of iodide, caused much of the iodine to leave the gland as iodide. Further, methimazole given several days after administration of a tracer dose of $^{131}I^-$ increased the specific activity of $^{131}I^-$ excreted in urine to 17% of administered $^{131}I^-$ per mg., whereas the specific activity in the gland was only 2.9% per mg. This iodide leaving the gland during methimazole treatment was derived from a small pool of labelled iodine of high specific activity. This pool was in the process of recycling iodine rapidly within the gland. In addition, there was a large and relatively sequestered pool of protein bound iodine in the gland. Examination of the thyroid four days after the administration of $^{131}I^-$ disclosed a large quantity of labelled iodotyrosines, but only trace amounts of stable thyroxine and no labelled thyroxine.

If this is indeed a defect in iodotyrosyl coupling it could arise by a deficiency in the activity of a hypothetical coupling enzyme or by a structural defect in thyroglobulin impairing coupling.

The absence of a dehalogenase has been put forward as a further cause of hypothyroidism explained in terms of an enzymic defect. Roche, Michel, Michel and Lissitsky (1952) and Roche, Michel, Michel, Gorbman and Lissitsky (1953) have discussed the role of deiodinases in allowing reutilization of iodine contained in the iodotyrosines freed after proteolysis of thyroglobulin. McGirr, Hutchison and Clement (1959) described a goitrous cretin whose thyroid lacked dehalogenase activity and whose thyroid tissue contained an abnormally high ratio of mono- to diiodotyrosine. The impaired ability to deiodinate monoiodotyrosine was found in other members of the family. Other workers (Querido, Stanbury, Kassenaar and Meijer, 1956) found large amounts of iodotyrosines, or products thereof, in serum of another patient. There are normally no iodotyrosines extrathyroidally. The nature of the defect was reinforced when (a) thyroid slices exhibited no deiodinating activity in contrast to the high level of activity in normal tissue, and (b) 50% of administered diiodotyrosine was excreted unchanged in the urine indicating a lack of extrathyroidal deiodinase systems as well.

Halmi and Pitt-Rivers (1962), by allowing rats to approach isotopic equilibrium on an $^{131}I^-$-containing diet over 3 wks., found a progressive build-up of the thyroid/serum $^{131}I^-$ ratio. Most of the radioiodide was not perchlorate-dischargeable after 24 hr. and they thought this pool was derived by deiodination of the iodotyrosines.

Quantitative labelling and kinetic studies of thyroidal iodine metabolism, using $^{131}I^-$, have led to complication of the postulated product-precursor relationship between mono- and diiodotyrosine. To explain the difference in the kinetics of iodination of tyrosine, as found by various laboratories, it has been suggested (Plaskett, Barnaby and Lloyd, 1963,a, and Stolc and Langer, 1963) that the

pools of iodoamino acids are heterogeneous.

At all times between 10 min. and 48 hr. after the injection of $^{131}I^-$ into rats Plaskett *et al.* (1963,a) found that the ratio of the activity of mono- to diiodotyrosine, R, was constant and there was a steady rise in the activity of thyroxine. This result, in agreement with Bois and Larsson (1958) and De Groot and Davis (1961,b) amongst others, was contrary to the findings of Pitt-Rivers and other workers where monoiodotyrosine displayed a product-precursor relationship with diiodotyrosine.

The constant ratio R would be compatible with a reaction between free tyrosine and iodine giving rise to an isotopically equilibrated pool for incorporation into thyroglobulin. This idea was rejected because of *in vitro* studies by Tong, Taurog and Chaikoff (1957) and because chromatography of unhydrolysed thyroid homogenates has not shown any significant change in ratio of labelled free iodotyrosines to labelled bound iodotyrosines from 10 min. to 2 hr. after injection of $^{131}I^-$ (Plaskett *et al.*, 1963,a). Labelled free iodotyrosines make up 0.3% of total activity of the gland and their specific activities are lower, at all times, than those of the protein bound iodotyrosines, thereby lending support to the idea they are derived from thyroglobulin and not *vice versa*.

A second possibility put forward was that particular tyrosyl residues could be mono- or diiodinated depending on their position or local environmental conditions, such as availability of a second iodine molecule after the first had reacted. This was the view of De Groot and Davis (1961,b) who speak of 'considerable specificity in the extent of iodination of tyrosyl groups in thyroglobulin'. Although there is most probably specificity of this kind, it is unlikely that rats from different laboratories should differ in the fundamental process of iodination and this idea was

not favoured as an explanation of a changing R value.

The most probable hypothesis depends on the iodotyrosine turnover being heterogeneous because of incomplete or selective proteolysis of thyroglobulin. After the iodination of the tyrosyl residues in thyroglobulin the newly iodinated protein can either follow the processes leading to iodothyronine synthesis and storage of the thyroglobulin in a large iodo-protein pool (I), or undergo proteolysis (II) with release of the iodotyrosines followed by deiodination. Further iodination of new uniodinated protein then occurs from this second, mixed radioiodine pool. The apparently contrary reports can be reconciled if pool I is large compared with pool II. If the second pool is small and rapidly turning over this will allow equilibration of the radioiodine atoms of mono- and diiodotyrosine. Whereas if the second pool was larger and less rapidly turning over the iodine atoms of diiodotyrosine synthesized by iodination of 'cold' monoiodotyrosine would have less chance to equilibrate with the more highly labelled iodine from newly iodinated monoiodotyrosine. This condition will give results as found by Pitt-Rivers (1962).

One point is clear, the large pool I containing all or most of the iodothyronines must for some reason undergo much less proteolysis than the newly iodinated protein containing mainly iodotyrosines. This can be brought about either by a radical change in shape, decreasing susceptibility to proteases, and/or by a spatial separation of the older protein from the newly synthesized material. This point will be discussed later in light of work on the neterogeneity of iodinated proteins in the thyroid.

Triiodothyronine comprises some 10% of the total iodothyronine fraction, a figure which varies according to the level of iodination.

It is thought to be synthesized by the coupling of mono- to diiodotyrosine. Thus if mono- and diiodotyrosine exhibit a product-precursor relationship the ratio of the specific activities of triiodothyronine and thyroxine should be raised soon after injection of $^{131}I^-$ when the ratio of the specific activities of mono- and diiodotyrosine is greater than one (Pitt-Rivers, 1962). However, Plaskett, Barnaby and Lloyd (1963,b) showed in their laboratory that triiodothyronine contained a radioactive contaminant, especially at early times, and that the ratio of activities of the iodothyronines was constant. As their ratio R was constant they could not show whether triiodothyronine was synthesized from mono- and diiodotyrosine or by the deiodination of thyroxine. Plaskett (1961) found, by investigation of the activities of the iodines in the 3 and 5 positions in thyroxine that it is not formed by iodination of triiodothyronine.

Plaskett (1963,b) wrote, 'An idea of the way in which the untenanted 5 position in triiodothyronine may escape iodination can be derived from the two-compartment mechanism of thyroid hormone biosynthesis proposed by Plaskett et al. (1963,a). Iodination and coupling probably occur in different fractions of thyroglobulin. If these fractions are spatially as well as functionally separated then no triiodothyronine would be present in the region of the follicle where iodination of tyrosyl residues occurs. Since iodination probably takes place in a region situated at the periphery of the follicles (Levenson, 1960) this explanation seems a reasonable one.'

As the synthesis, iodination and heterogeneity of thyroglobulin are central to the present study, the second part of the introduction will deal mainly with the present views on various aspects of

thyroglobulin.

The sequence of events leading to thyroglobulin synthesis has been studied by administration of ^{3}H- or ^{14}C-amino acids, usually leucine, and followed by electronmicroscopy combined with autoradiography. A thyroid follicle is composed of a single layer of epithelial cells arranged in a sphere with the basal part of the cells on the outside and the apices of the cells facing toward the middle of the follicle surrounding the colloid-filled lumen. Amino acids, taken up from the blood capillaries surrounding each follicle through the base of the epithelial cells, are distributed evenly over the cell cytoplasm. In 10 min. the activity passes to the endoplasmic reticulum and from there to the supra-nuclear Golgi region (Nadler, 1965) nearer to the apex of the cell. A protein which was bound to the particulate fraction of thyroid cells has been isolated (Spiro and Spiro, 1965) and has been shown to have the same carbohydrate content and immunological and electrophoretic properties as soluble thyroglobulin. The 8% carbohydrate content of thyroglobulin is made up of about 300 monosaccharide residues arranged in approximately 23 units of 2 types which are thought to be attached after peptide formation. This process probably occurs in the region of the Golgi apparatus. Puromycin, an inhibitor of protein synthesis at the ribosomal level, inhibits the incorporation of ^{14}C-leucine into thyroidal protein by 95-99% (Maloof, Sato and Soodak, 1964). In contrast, the rates of uptake of ^{14}C-glucose into the monosaccharide residues in the presence of puromycin were less inhibited. The degree of inhibition noted in the various sugars was related to their sequential arrangement in the carbohydrate units, suggesting that there is a sequential attachment of the monosaccharides to the oligosaccharide chains during biosynthesis.

From the Golgi region the ^{14}C- or ^{3}H-labelled protein migrates as vesicles to the apical regions of the cells. After about 4 hr. the protein appears in the periphery of the lumen of the follicle - the so-called ring reaction. The width of this ring of labelled protein is 7.5 (Simon and Droz, 1965) and the protein is estimated as leaving the cell apices at about 3 per hr. After 7 hr., even in the larger follicles, the protein has diffused throughout the colloid. Treatment with thyrotrophic hormone causes streamers of cytoplasm to invade the periphery of the colloid with the removal of colloid as droplets. These droplets become associated with the lysosomes and there follows, presumably, hydrolysis of the thyroglobulin and release of iodothyronines.

In contrast to the time scale of synthesis and movement of the newly synthesized thyroglobulin the iodination process is very rapid. Labelling with $^{125}I^-$ for very short periods followed by autoradiography (Nadler, 1965) showed that after 30 sec. 95% of the radioactivity had passed into the colloid. Simon and Droz (1965) showed that, after longer intervals there were 6 times as many autoradiogram grains over the lumen as there were over the cells, and the latter reaction was mainly from colloid droplets presumably containing $^{125}I^-$ protein from the lumen. Spiro and Spiro's (1965) protein isolated from the cell particulate fraction contained very much less iodine than did the thyroglobulin isolated from the mixed cell cytoplasm and colloid (produced by homogenization) or medium in which the cells had been incubated.

It appears that iodination takes place mainly or entirely in the periphery of the follicle. Simon and Droz (1965) found the iodinating system confined to this area with the highest concentration near the apices of the cells. A further study of this by

Nadler (1965) involving pre-treatment of the tissue slices with thyrotrophic hormone revealed that none of the colloid thyroglobulin present as intracellular vesicles following pinocytosis was iodinated by passage of $^{125}I^-$ through the cell.

The newly synthesized thyroglobulin, because of its relatively slow speed of diffusion away from the periphery of the follicular lumen, is the protein fraction most likely to be subject to proteolysis either by enzymes released from the cells or by being engulfed by the cytoplasm.

The iodotyrosines released on hydrolysis will be deiodinated by the microsomal deiodinases and the iodide will be returned rapidly to the lumen. The existence of two fractions of thyroglobulin; one near to the periphery of the follicle and the other near to the middle of the follicle, might give a reality to the two-compartment model of Plaskett (1963,a) and also account for the slowness of equilibration of $^{131}I^-$ into thyroid (Halmi and Pitt-Rivers, 1962) which is a measure of the slowness of equilibration of the larger slow pool I.

Iodination of thyroglobulin

The iodinated proteins from thyroid can be separated by ultracentrifugation into groups with different sedimentation constants, namely, 3-8S, 12S, 19S, 27S and 31-33S. After the initial work of Seed and Goldberg (1963) it is now fairly well established that the 12S fraction is a precursor of the 19S and under certain conditions 19S can dissociate into 12S. It is not yet quite clear, however, whether the 12S produced by the dissociation of 19S is the same as the 12S precursor. The larger proteins are polymers of 19S with or without the addition of 12S or even smaller protein units.

There is little evidence for the 3-8S fraction being a precursor of thyroglobulin. Ultracentrifugally pure thyroglobulin can be separated on carboxymethyl cellulose into fractions with different iodine to nitrogen ratios. There may also be heterogeneity with respect to carbohydrate content. 'Thyroglobulin' is defined as the iodinated thyroidal protein precipitating between 37 and 41% saturated ammonium sulphate and having a Svedberg constant of 19S. The newly synthesized 'thyroglobulins' tend to be lighter (17S) because of a lesser degree of iodination or incomplete intramolecular rearrangement during or following coupling.

Synthesis and polymerisation can occur without iodination. It is unlikely that protein synthesis depends on the iodide content as the latter is very variable. Puromycin blocks protein synthesis but not iodination (Maloof et al., 1964). By centrifugation on a sucrose gradient soluble protein, synthesized by a cell free protein-synthesising system from thyroid, is found to contain 17S-type proteins. When 'new' thyroglobulin, labelled with ^{14}C-tyrosine, is centrifuged with pure $^{125}I^-$ thyroglobulin as a standard the new protein has a sedimentation coefficient of 17S. The horse radish peroxidase-glucose-glucose oxidase system (Edelhoch, 1962) iodinates thyroglobulin to a high level and increases the sedimentation coefficient to greater than 19S. Sena, Viscidi and Salvatore (1965) carried out analyses for $^{127}I^-$ in 12S, 19S and 27S fractions. They found that iodine, expressed as a percentage of protein dry weight, was very low in 12S and higher in 27S than in 19S. Uptake of $^{131}I^-$ was most rapid into the 12S fraction whose activity then fell with time as the levels of $^{131}I^-$ in 19S and 27S rose. Thyrotrophic hormone treatment speeded up the uptake and loss of iodide but did not affect levels of iodine in the fractions.

Specificity of iodination

The tyrosyl residues in thyroglobulin can be iodinated randomly or some specific residues can be iodinated. Casein *in vitro* can be highly iodinated giving rise to iodotyrosines and thyroxine. Is this type of mechanism feasible for thyroglobulin?

There are 125 tyrosyl residues in sheep thyroglobulin, of which 9 are monoiodotyrosine, 6 are diiodotyrosine and 3 are thyroxine (Lissitsky, 1966): the latter probably from 6 diiodotyrosines. Of the original 125 tyrosyl residues, 21 have been iodinated to some extent giving 18 iodoamino acid residues. Edelhoch (1962) has shown that 20% of the residues in hog thyroglobulin are not accessible for iodination. Further, *in vitro* iodination to an extent greater than 40% of the total possible leads to structural changes in the protein as shown by increased sensitivity to temperature, pH and ionic strength. *In vivo* the total number of tyrosyl residues for iodination must be not greater than 40% of 125, i.e. 50 residues. Of this 50 only 21* will in fact be iodinated in each molecule. Each tyrosyl residue may, if iodination is random, be uniodinated, monoiodinated or diiodinated, thus making a maximum 21 residues per molecule which could be labelled with radioiodine. Digestion of the protein with α-chymotrypsin, which attacks on the carboxyl side of the aromatic residues, will release from a thyroglobulin preparation a possible 21 labelled peptides per molecule but a total of 2 x 50 labelled petides from the preparation. Assuming that the iodinated tyrosyl residues are drawn from the same 50 in each case, the total number of labelled peptides from a

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* Neglecting the conversion of 6 diiodotyrosine residues to thyroxine which *in vitro* is negligible.

^{14}C-tyrosine labelled thyroglobulin would be the remaining 75 uniodinated tyrosyl peptides plus the peptides containing the tyrosyl residues that can be uniodinated, monoiodinated or diiodinated, thus making a total of 225 in all.

If iodination is not random, and we have already seen that 25 of the tyrosyl residues cannot be iodinated and a further 50 cannot be iodinated without structural changes, then certain of the tyrosyl residues must be more easily iodinated than others. Whether these residues form mono- or diiodotyrosine may depend on their specific properties or merely on the availability of iodine. It is known that the level of iodine in the diet may affect the ratio of mono- to diiodotyrosine but if one tyrosyl is more easily iodinated than another it will under any given conditions be more readily converted to diiodotyrosine than another residue which may remain as monoiodotyrosine. If iodination is specific only 21 different iodopeptides should be released from <u>in vitro</u> iodinated thyroglobulin by α-chymotrypsin.

Specificity could theoretically arise in three ways: (i) only certain residues are available for iodination (De Groot and Davis, 1961,b); (ii) there is a specific iodinase which can react with a restricted number of the tyrosyl residues; (iii) the situation of some of the tyrosyl residues in relation to other residues in the protein creates 'active sites' for iodination.

As only 21 of the 50 tyrosyl residues not apparently involved in the secondary or tertiary structure of sheep thyroglobulin are iodinated the accessibility of iodine to these residues does not seem a sufficient reason for their specificity of iodination.

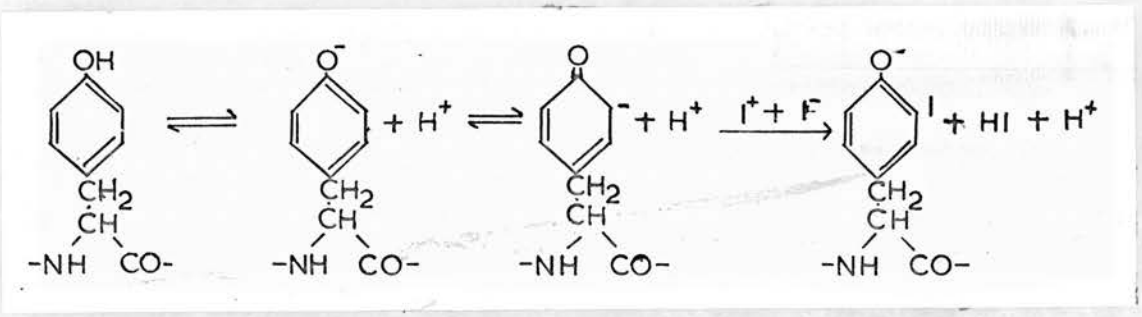
The second of these possibilities depends on the existence of an iodinase, that is, an enzyme which in the presence of iodide

and tyrosyl residues will specifically iodinate the latter. Such an enzyme has not been demonstrated although thyroid contains more than one peroxidase which can, in the presence of hydrogen peroxide, oxidise iodide to iodine. Artificial peroxidase iodinating systems can, by converting iodide to iodine, iodinate many more tyrosyl residues with no specificity residing in the enzyme system. However, at physiological levels of icdide and enzyme there may be just enough iodine produced to iodinate only the most susceptible of the tyrosyl residues.

This leads to the third possibility, that the specificity resides in the situation of the tyrosyl residue producing what might be called an 'active site' for iodination. The iodination is thought to take place as an electrophilic attack by an iodinium ion on the phenoxide radical.

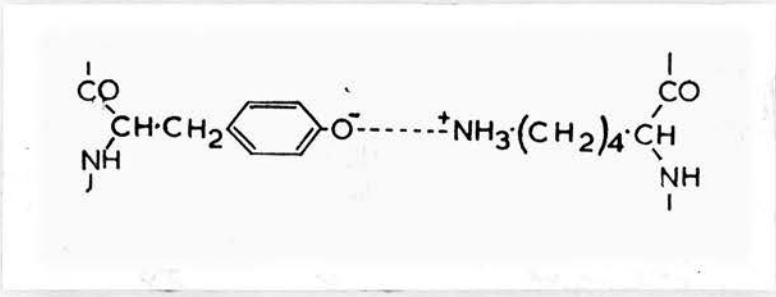
$$2I^- \longrightarrow I_2 + 2\epsilon^-$$

$$I_2 \longrightarrow I^+ + I^-$$



The oxidation of iodide to iodine is thought to be mediated by one or more thyroidal peroxidases (Alexander, 1965) and the iodine is considered to be the iodinating species. Because of its large size the iodine molecule will not be available to all tyrosyl residues and in some cases iodination may not proceed further than the monoiodination stage because of steric effects.

The pK of the phenolic hydroxyl is 10.2 and only 0.1% of the tyrosyls will be dissociated at the pH of the cytoplasm. Perhaps the formation of a salt linkage, either inter- or intrachain, with a positively charged group in the vicinity would increase dissociation.



Once iodination has occurred further specificity may be involved in the coupling of two diiodotyrosyl residues giving thyroxine. Of the presumed 12 diiodotyrosines produced, 6 are coupled to form 3 thyroxyl residues. This 50% yield is unlikely to occur by random coupling in thyroglobulin. Two diiodotyrosyl residues may arise in proximity by the iodination of two tyrosyl residues which are close together on a peptide chain or which can be juxtaposed by the crossing over of two separate peptide chains. The diiodotyrosyls are probably not brought together by the dimerisation of 12S units as 17S protein has been isolated in an uniodinated state from cell free protein synthesizing systems and other 17S protein from cell slices (Mauchamp, Macchia and Nunez, 1965) has been shown to be 'the non-iodinated immediate precursor of the more or less halogenated protein family defined as thyroglobulin'. It appears likely that the maturation of 17S to 19S protein occurs with, or is caused by, iodination and subsequent thyroxine formation.

In the absence of evidence for a specific iodinase it seems most likely that if there is specificity of iodination it is dependent on the differences in the structural environments of the

different tyrosyl residues. Both questions will be resolved if it can be shown that the tyrosyl residues which are iodinated occur in a different structural environment from those which are not.

Objects of research

(a) To investigate the specificity of iodination, that is, to discover whether only a limited number of tyrosyl residues in thyroglobulin are iodinated;

(b) To isolate and partially characterise the parts of the peptide chains in which the iodinated tyrosyl residues were found;

(c) Isolation of an uniodinated thyroglobulin from a cell free system followed by controlled *in vitro* iodination to find if two diiodotyrosyl residues disappear during iodination with the formation of a thyroxyl residue.

Outline of research to achieve above objects

The research falls into six phases. Part I deals with the isolation and characterisation of a cell free protein synthesizing system from rat thyroid. This is followed in Part II by investigation of the protein synthesizing systems in whole thyroids from rat or sliced thyroids from sheep. These systems, especially the latter, give good yields of thyroglobulin containing high levels of ^{14}C-amino acids. The purity of the isolated thyroglobulin both with respect to radioactivity and protein was checked and analyses for amino acid and iodoamino acid content were carried out (Part III).

Thyroglobulin, iodinated *in vitro*, was isolated from the above slice preparation (Part IV). The α-chymotrypsin hydrolysate of this was peptide mapped and the labelling patterns of the two isotopes compared in an effort to identify either the thyroxine peptide or

peptides containing high levels of diiodotyrosine.

This was followed by the isolation of individual iodoamino acid-containing peptides and investigation of their molecular weights, iodoamino acid contents and kinetics of iodination (Part V).

Chemical iodination (Part VI) was used to determine the quantity of certain peptides in thyroglobulin and, by altering peptide mobilities, to examine the relationship between the mono- and diiodotyrosine-containing peptides.

Chapter 1

ISOLATION AND CHARACTERISATION OF A CELL FREE PROTEIN SYNTHESIZING SYSTEM

Cell free protein synthesizing systems have been isolated from many sources including bacteria, plants and animals, but before this work was started there had been no reports of the isolation of such a system from thyroid. It was appreciated that the isolation of this type of preparation presented some difficulties but that these could be overcome by familiarization with the necessary techniques while isolating a relatively well characterised cell free system. Isolation of the protein synthesizing system from rat liver was felt to be a necessary prelude to a similar treatment of thyroid tissue and it was thought that this would allow recognition of any special problems which might arise with the latter tissue.

It was hoped that application to thyroid tissue of the techniques developed with rat liver would yield an active protein synthesizing system. Further investigation was planned to determine if the thyroid system differed from other comparable systems and, if possible, to increase the specific radioactivity of the isolated protein.

I.1. Sources of tissues

Livers and thyroids were usually taken from male Wistar albino rats of 200-300 g. body weight which had been starved overnight. The animals were lightly anaethetised with ether and killed by exsanguination following heart puncture. The required tissue was

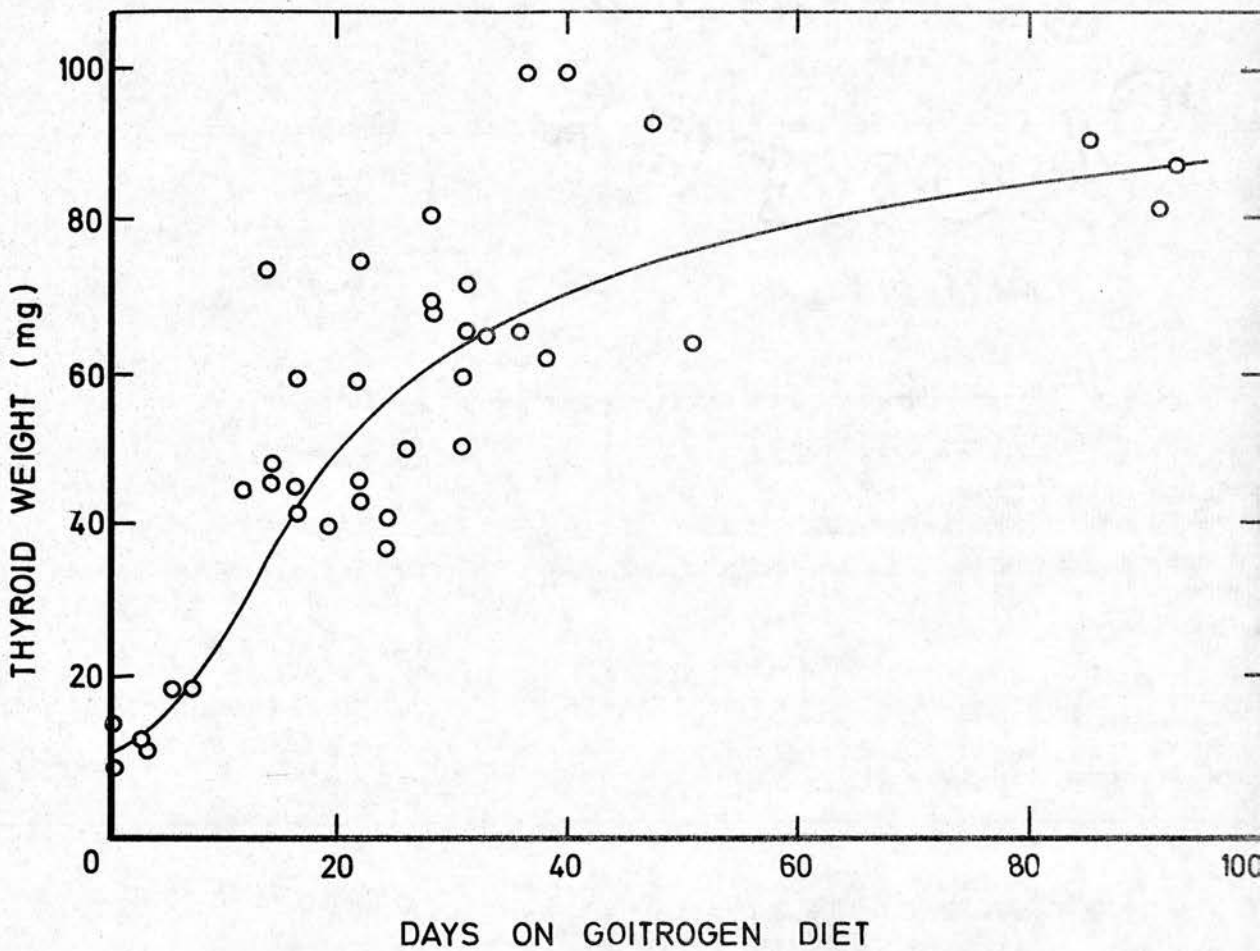


FIGURE 1 EFFECT OF GOITROGEN ON THYROID WEIGHT.

immediately removed, cooled by chopping with scissors in ice-cold medium and its weight found by difference. Livers weighed from 7-10 g. and thyroids from 15-20 mg. per animal.

To increase the thyroid size, 0.6% by weight of 2-thiouracil (TU), or 0.2% by weight of 4-n-propyl-2-thiouracil (PTU), or 4-n-methyl-2-thiouracil (MTU) was added to the animals' low-iodine stock diet (Boyd and Oliver, 1960). These goitrogens increased the average thyroid size to 50 mg. in 20 dy. and over 100 mg. in 3 mth. (Fig. 1). Most of the animals were exposed to a goitrogen for a minimum of 2 wk. and were sacrificed soon after.

Thyroid tissue was occasionally procured from guinea-pigs which had been fed goitrogen either in capsule form or by adding TU and ascorbic acid to their drinking water.

These animals were much more sensitive to the goitrogen and could not be maintained on it without their condition deteriorating. The weight of thyroid ranged from 18-77 mg.

Ox, hog or sheep thyroids were transported from the local slaughterhouse in polythene bags packed in ice-filled vacuum flasks. The time between the death of the animal and homogenization of the gland was about 1 hr.

Ox thyroids weighed approximately 40 g., hog 6 g. and sheep 2 g. each.

All dissection, trimming and slicing of tissue was done in the cold room at 2-6°. At other times the preparations were kept in ice.

I.2. Methods

I.2.1. Tissue homogenizers: Homogenizers were of the Potter-Elvehjem type fixed in ice-bottles to minimise the heating effect of homogenization. Their capacities ranged from 5 ml. to 20 ml. The

Table 1. Buffered media used during the preparation of cell-free amino acid incorporating systems

Constituents μ moles/ml.	Media I*	II*	III+	IIIa×	IIIb	IIIc‡	IIId	IV+
Tris	30	45	-	50	-	-	-	-
$NaHCO_3$	-	-	35	-	35	-	-	-
$KHCO_3$	-	-	-	-	-	35	35	-
KH_2PO_4	-	-	20	20	4	4	4	-
Na_2HPO_4	-	-	-	-	16	-	16	-
K_2HPO_4	-	-	-	-	-	16	-	-
$MgCl_2$	5	7.5	4	4	4	6	6	4
KCl	80	120	25	25	25	25	25	25
NaCl	50	75	-	-	-	-	-	-
Sucrose	-	-	350	350	350	350	350	900
Na^+	50	75	35	0	67	0	32	0
K^+	80	120	45	44	29	96	64	25

* Korner (1962)

\+ Keller and Zamecnik (1956)

× Zamecnik and Keller (1957)

‡ Allen and Schweet (1962)

usual combination of Teflon ~~tube~~ pestle and Viridium glass ~~pestle~~ tube was discarded in favour of all-glass power-driven homogenizers with toothed pestles, as only these were capable of disrupting thyroid tissue.

I.2.2. Homogenization: The medium in which the tissue had been cooled and minced was drained off and the tissue was added to the homogenizer with a suitable quantity of ice-cold homogenization medium. Although in initial experiments the homogenization medium was 0.44M sucrose, in later experiments the medium was usually medium III (Table I) or modifications of this, (media IIIa to IIId). Preliminary washing removed much blood leached from the tissue during mincing. Three or four strokes of the pestle at slow speed were usually enough to disrupt most of the tissue. Dilution of the homogenate with the first dilution medium was done at this stage, followed, where necessary, by a second dilution with a mixture of the homogenization medium and the first dilution medium. Where 0.44M sucrose had been used as the homogenization medium, I or II was used as the first dilution medium. Latterly, with homogenization in medium III, IV was used as the first dilution medium.

I.2.3. Preparation of centrifuged homogenate: The homogenate was centrifuged at 15,000 g for 20 min. at 0-2° in an MSE 17,000 centrifuge. By pipetting off the top three-quarters of the supernatant the centrifuged homogenate was isolated free from floating lipid, sub-cellular particulate fractions, except the microsomes, and cell debris. In some cases further dilution was done at this point.

I.2.4. Preparation of microsomes and cell sap: To sediment the microsomes the centrifuged homogenate was spun at 105,000 g for 1 hr. or at 125,000 g for 40 min. at 0-2° in a Spinco Model L Preparative Centrifuge. The post-microsomal supernatant, or cell sap, was

decanted. The microsomal pellet was rinsed with the same mixed medium from which it had sedimented and was then resuspended by hand in the same medium. After centrifugation again the supernatant was discarded, the tube was rinsed with incubation medium and the pellet was carefully resuspended by hand in the same medium using a small glass homogenizer.

I.2.5. Preparation of 'pH 5-enzymes': The method used was that of Hoagland, Keller and Zamecnik (1956). The pH of the ice-cold cell sap was lowered from pH 7.6 to pH 5.2 by the drop-wise addition of ice-cold 0.1M acetic acid. After standing at 0° for 10 min. the suspension was centrifuged at 2,000 *g* for 10 min. at $0-2^{\circ}$, the supernatant was discarded, the tube was rinsed with IV, and the protein pellet was washed by resuspension and resedimentation from IV. Finally, the tube and pellet were rinsed with incubation medium and the protein was taken up in a small volume of the buffer to give a solution of approximately 8 mg./ml. Where necessary the final solution was centrifuged to remove undissolved protein. Occasionally a sample of the 'pH 5-enzyme' solution was subjected to the purification procedure outlined below to check the protein concentration.

I.2.6. Preparation of ribosomes: (Korner, 1962) To the centrifuged homogenate was added one-ninth its volume of a 5% solution of deoxycholate in 0.03M Tris buffer at pH 8.2 giving a final concentration of 0.5% deoxycholate. The solution was centrifuged at 105,000 *g* for 2 hr. at $0-2^{\circ}$. After decanting the supernatant, and drying the tube with paper tissue, the ribosomal pellet was resuspended in twice the supernatant volume of incubation medium. Recentrifugation as above for 90 min. produced a pellet of washed,

ribosomes which was resuspended in one volume of incubation medium.

I.2.7. Incubation of complete system: Each incubation contained, in 1.0 ml., 0.75 ml. of centrifuged homogenate; or 0.5 ml. microsomal suspension with 0.25 ml. of cell sap; or 0.5 ml. microsomal or ribosomal suspension with 0.25 ml. of 'pH 5-enzyme' solution. Added to these, in 0.25 ml. of incubation medium, were 10 μmoles of creatine phosphate, 1.0 μmole of ATP, 0.25 μ mole of GTP and varying quantities of L-leucine-^{14}C(U). The pH of this latter solution was adjusted to 7.5-7.7 with 1M KOH and 0.03 mg. of creatine phosphokinase added. The combined solutions were incubated at 37° for 1 hr. in air, unless otherwise stated, and the reaction stopped by the addition of 2 ml. of 10% trichloracetic acid. If the microsomes and soluble enzymes were to be separated prior to their precipitation with trichloracetic acid, 10 ml. of ice-cold 0.44M sucrose containing a ten-fold excess of stable leucine was added and the suspension centrifuged at 105,000 *g* for 1 hr. at 0-2°. The supernatant was decanted, the tube was rinsed with buffer and the washing was added to the supernatant. Both this and the microsomal fraction were precipitated in trichloracetic acid of a final concentration of 6-7%.

I.2.8. Measurement of incorporated radioactivity: The trichloracetic acid-precipitable protein was washed three times with 2 ml. of 0.5N perchloric acid containing a ten-fold excess of unlabelled leucine and boiled in 2 ml. of the same solution. The protein was then dissolved in 2 ml. of 1N NaOH to extract ribonucleic acid, reprecipitated 1 hr. later with 6N HCl, washed twice with chloroform:ether:ethanol (1:2:2, by volume) and dried with ether.

The dry protein powder was plated on to weighed aluminium planchets (14 mm. in diameter) with ether. After evaporation of

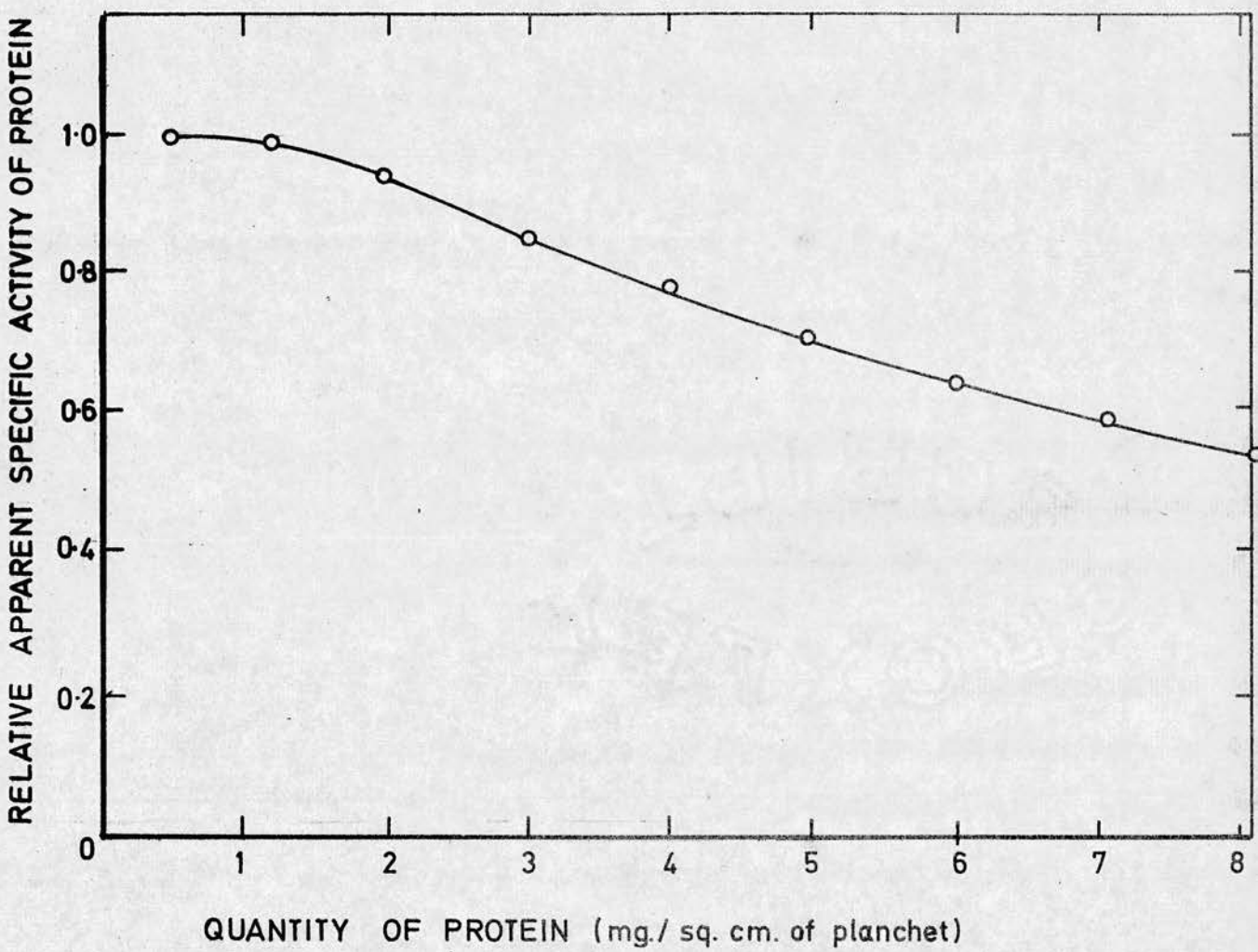


FIGURE 2 SELF ABSORPTION OF THE β-EMISSIONS FROM ^{14}C-LABELLED PROTEIN.

the ether, and reweighing of the planchet, the radioactivity of the protein was assayed by a Geiger-Müller end window counter, type EHM2S connected to an Ekco Scaler type N530G with a pre-amplifier, and the incorporated radioactivity was expressed as a percentage of the added L-leucine-^{14}C(U) per mg. of dry protein. This counting method was 7% efficient. Corrections for self-absorption, when the amounts of protein were small and of comparable weight, could be neglected. Larger samples of protein were corrected for self-absorption from a standard curve (Fig. 2) which had been compiled by plating and counting increasingly large samples of protein of different specific activities. Below 1 mg. of protein per sq. cm. the self-absorption was constant. Uneveness of plating was difficult to control or measure and was a source of counting error. When a large quantity of protein had been isolated only a fraction of this, approximately 4 mg. in weight, was plated and counted. The remainder of the protein was then plated on to the same planchet which was reweighed to give the total weight of protein. In this way, inaccuracies in counting from self-absorption and plating were minimised.

Each batch of incubations included a control tube where the reaction had been stopped at time zero by the addition of 2 ml. of trichloracetic acid or by heating the tube in a boiling water bath for 5 min. The protein from this tube was isolated, along with the protein from the other incubations, and any counts registered by this planchet were subtracted from the activity of the other protein samples. Casein solution of known concentration was sometimes added to the cooled incubation immediately before precipitation with trichloracetic acid when the quantity of microsomal and 'pH 5-enzyme' protein was low. The loss of protein during purification was up to 46%.

Table 2. Incorporation of ^{14}C-leucine by rat liver centrifuged homogenate

Centrifuged homogenate (0.75 ml.) was added to 0.25 ml. of media II, (a); I, (b) and (c) or III, (d) containing 3 μmoles of ATP, 10 μmoles of creatine phosphate, 1 μmole of GTP, 0.03 mg. of phosphocreatine kinase and 0.5 μC of ^{14}C-leucine

Experiment	Homogenization medium	Dilution medium	Time of incubation (min.)	Incorporation† (%/mg.)	Cations in incubation medium* Na^+ (μmoles/mg.)	K^+ (μmoles/mg.)	Mg^{++} (μmoles/mg.)
a	0.44M sucrose	II	30	0.019	69	84	5.3
			60	0.028	69	84	5.3
b	0.44M sucrose	II**	15	0.0056	47	56	4.6
			30	0.0063	47	56	4.6
c	0.44M sucrose	II	60	0.027	62	76	4.6
			60	0.028	62	76	4.6
d	III	IV	60	0.032	36	37	4.9

* These values include cations from the salts of ATP, GTP and creatine phosphate

† Percentage of ^{14}C-amino acid in incubation incorporated per mg. dry protein

** In experiment (b) the dilution medium was added after centrifugation of the homogenate. In experiments (a), (c) and (d) it was added before centrifugation

I.3. Results of incorporation of ^{14}C-leucine into rat liver preparations

The first attempts at isolating an amino acid incorporating system from rat liver followed a procedure published by Korner (1962). In this, the homogenization medium was 0.44M sucrose and the microsomal and ribosomal fractions were washed in medium I and resuspended in medium II (Table 1) for incubation. To 0.5 ml. of these were added 0.25 ml. of cell sap and 5.0 μmoles ATP, 0.6 μmoles GTP, and 0.5 μC ^{14}C(U)-leucine in 0.25 ml. of medium I.

Maximally, only 0.006%/mg. of the ^{14}C(U)-leucine was incorporated into trichloracetic acid precipitable protein after incubation of these ribosomal or microsomal preparations.

Using the microsomal-cell sap preparation it was impossible to tell whether the loss of activity occurred at the homogenization stage or during a later part of the preparation. Checking the incorporation at each stage of the preparation revealed where the loss of activity was occurring.

I.3.1. Incorporation into centrifuged homogenate: Early experiments with amino acid incorporating systems had made use of the centrifuged homogenate preparation to investigate both isolation procedures and media. An early method of preparation (Zamecnik and Keller, 1954) was modified and used to prepare centrifuged homogenate.

Homogenization of rat liver in two volumes of 0.44M sucrose was followed immediately by dilution with three volumes of medium II. Incorporation, after incubation under the conditions in Table 2:(a), compared favourably with the 0.023%/mg. incorporation quoted by Zamecnik and Keller (1954) in their paper.

From the ^{14}C-leucine uptake it appeared that the homogenization, which was unaltered from the previous experiments, was not the cause

Table 3. Incorporation of ^{14}C-leucine by rat liver microsomal-cell sap preparation

The incubations contained 0.5 ml. of microsomal suspension in 0.44M sucrose:medium II (2:3, by volume), 0.25 ml. of cell sap and 0.25 ml. of medium I containing the same additions as noted in Table 2.

Time of incubation (min.)	Incorporation (%/mg.)	Cations in incubation medium		
		Na^+	K^+	Mg^{++}
		(µmoles/ml.)		
60	0.057	62	76	4.6
60	0.061	62	76	4.6

of the loss of activity. The preparation remained capable of amino acid uptake for longer than 30 min.

In a second experiment of the same type the dilution medium, in this case I, was not added until the homogenate had been centrifuged. The loss of activity, Table 2:(b), might logically be attributed to the delay in adding the ionic medium or to the lowered ionic strength of the final incubation medium.

To investigate this idea a second centrifuged homogenate was prepared under the conditions given above (foot of p. 31). This was found to be as active as the earlier preparation, Table 2:(c).

Homogenization of rat liver in medium III (2.3 vol./g. tissue) instead of 0.44M sucrose was followed immediately by dilution with 3 vol. of IV. The centrifuged homogenate was incubated under the conditions in Table 2:(d). The raised activity indicated a requirement for some or all ionic constituents of the media during or immediately after homogenization.

I.3.2. Incorporation using the microsome-cell sap preparation: Using the earlier conditions (foot of p. 31), previously found to be successful, centrifuged homogenate was prepared and the microsome fraction was isolated, as outlined in Methods (I.2.), from some of this.

The microsomes were resuspended in sucrose:medium II (2:3, by volume), and the concentration adjusted so that all incubations contained the same quantity of microsomes. Microsome-cell sap preparations, under the conditions given in Table 3, yielded a protein twice as highly labelled as that isolated from the incubations containing centrifuged homogenate. The results of the incubations with centrifuged homogenate from which the microsomes were

Table 4. Incorporation of ^{14}C-leucine by rat liver centrifuged homogenate after dilution

Each incubation comprised 0.75 ml. of centrifuged homogenate and 0.25 ml. of medium III : medium IV (2.3 : 3, by volume), containing 10.0 µmoles of creatine phosphate, 1 µmole of ATP, 1 µmole of GTP, 0.03 mg. of phosphocreatine kinase and 0.5 µC of ^{14}C-leucine. Incubation for 60 min.

Dilution	Incorporation (%)	Protein isolated (mg.)	Specific activity of protein (% of ^{14}C-leucine added/mg. dry protein)
None	0.676	27.8	0.024
1:4	0.136	7.9	0.017
1:8	0.095	5.0	0.019
1:16	0.048	2.4	0.020

Table 5. Incorporation of ^{14}C-leucine by rat liver microsomal-'pH 5-enzyme' preparations in the presence and absence of an ATP-generating system under air, oxygen or nitrogen

Incubations contained, in medium III, 0.5 ml. of microsomal suspension, 0.25 ml. of 'pH 5-enzyme' solution and, in 0.25 ml., 0.5 µC ^{14}C-leucine with or without a source of ATP.

Gas phase	ATP-generating system	Specific activity of protein* (% of ^{14}C-leucine added/mg. dry protein)	Inhibition (%)
Air	−	0.0045	84
	+	0.028	
O_2 : CO_2 (95% : 5%)	−	0.0035	88
	+	0.029	
N_2 (100%)	−	0.0030	84
	+	0.019	

* Mean of duplicates

prepared were quoted earlier in Table 2:(c).

I.3.3. Incorporation into diluted homogenates: In order to obtain workable volumes of microsomal and cell sap preparations from small quantities of thyroid tissue it was necessary, at some stage, to dilute the homogenate. The tissue to medium ratio during homogenization is apparently fairly critical, according to several workers, and dilution was carried out immediately after homogenization, that is, after the homogenization medium and disrupted tissue had the first dilution medium added. The second dilution medium was always a mixture of the homogenization medium and the first dilution medium in the ratio in which these had been added for homogenization and for dilution afterwards respectively.

A centrifuged homogenate of rat liver, prepared using media III and IV, was diluted four-, eight- and sixteen-fold and incubated under the conditions given in Table 4. Apart from a slight variation the specific activities of the isolated protein were constant. The weights of protein isolated did not correspond to the dilutions, but this was due to losses of protein from the samples during purification.

I.3.4. Energy dependence of the microsome-'pH 5-enzyme' system under air, oxygen and nitrogen: Microsomes and 'pH 5-enzyme' were prepared using media III and IV and taken up in medium III before incubation. The ATP-generating system, GTP and ^{14}C-leucine were dissolved in 0.25 ml. III. In the absence of the ATP-generating system only the ^{14}C-leucine was added in 0.25 ml. medium.

Portions of the incubation medium, III, had been equilibrated with air, oxygen:carbon dioxide (95%:5%) and nitrogen before use. During incubation the tubes not open to the air had a slow stream of the gases entering and leaving, ensuring a constant gas phase.

Table 6. Incorporation of ^{14}C-leucine by rat thyroid microsomal-cell sap preparations

Microsomal suspension (0.5 ml.), cell sap (0.25 ml.) and the ATP-generating system with 0.5 μC ^{14}C-leucine (in 0.25 ml.) taken up in medium III : medium IV (2.3 : 3, by volume). Where the centrifuged homogenate was substituted for the microsomal-cell sap system, 0.75 ml. was added.

Preparation	Incorporation (%)	Protein isolated (mg.)	Specific activity of protein (% of ^{14}C-leucine added/mg. dry protein)
Centrifuged homogenate	0.040	6.3	0.0063
Microsomal-cell sap	0.137	2.8	0.049
Centrifuged homogenate	0.082	4.3	0.019
Microsomal-cell sap	0.154 0.158	2.1 2.2	0.073 0.072

Table 7. Incorporation of ^{14}C-leucine by rat thyroid microsomal-'pH 5-enzyme' preparation

All fractions taken up in medium III : medium IV (2.3 : 3, by volume). Microsome suspension (0.5 ml.), 'pH 5-enzyme' solution (0.25 ml.) and the ATP-generating system with 0.5 μC ^{14}C-leucine in 0.25 ml.

Time of incubation (min.)	Incorporation (%)	Protein isolated (mg.)	Specific activity of protein (% of ^{14}C-leucine added/mg. dry protein)
0	0.015	2.8	0.0054
20	0.078	2.5	0.031
40	0.142	2.0	0.071
60	0.220	3.0	0.073

The gases were saturated with water vapour by bubbling them through distilled water in closed flasks kept at 37° in the same water bath. Duplicate incubations were set up (Table 5).

Under the three different gas phases the incorporation was dependent, to over 80%, on an energy supply.

The isolation procedure was capable of producing from rat liver an amino acid incorporating system whose properties, where tested, were the same as those reported by other workers. Application of this procedure to rat thyroid was expected to yield a similar amino acid incorporating system.

I.4. Results of incorporation of ^{14}C-leucine into rat thyroid preparations

I.4.1. Incorporation by centrifuged homogenate: A centrifuged homogenate of rat thyroid was prepared using media III and IV. Further dilution was done with a mixture (2.3:3, by volume) of these. According to the quantity of tissue available and the number of incubations necessary, further dilutions were from eight- to fifteen-fold.

Incorporations into the centrifuged homogenate ranged from 0.006%/mg. to 0.031%/mg. with the majority between 0.011% and 0.019%/mg.

I.4.2. Incorporation by microsome-cell sap preparations: Microsomes and cell sap were prepared with media III and IV. Incubations were set up under conditions identical to those for liver preparations (Table 3). Incubations containing centrifuged homogenates were included to ~~determine~~ compare the ~~increased~~ activity of the recombined preparations. Table 6 shows that the incorporation was increased between four to eight times. The results for thyroid were better

Table 8. Incorporation of ^{14}C-leucine by rat thyroid microsomal-'pH 5-enzyme' preparation with increasing ratios of 'pH 5-enzyme' protein to microsomal protein

All fractions taken up in media III and IV and incubated under the usual conditions.

'pH 5-enzyme' solution (ml.)	Medium III/IV (ml.)	Incorporation (%)	Protein isolated (mg.)	Specific activity of protein (% of ^{14}C-leucine added/mg. dry protein)
0.0625	0.1875	0.123	2.3	0.054
0.125	0.125	0.163	3.3	0.049
0.250	-	0.204	5.8	0.035
Centrifuged homogenate		0.125	4.1	0.030

Table 9. Effect of (a) casein hydrolysate, (b) complementary amino acids, and (c) ^{12}C-leucine on incorporation of ^{14}C-leucine by the rat thyroid microsomal-'pH 5-enzyme' preparation

Incubations under standard conditions. (Table 7.)

(a)

Casein hydrolysate (µg.)	Specific activity of protein (% of ^{14}C-leucine added/mg. dry protein)
0	0.160
3.8	0.121
38.0	0.104
380	0.114
380	0.098

(b)

Complementary amino acids (mole/mole ^{14}C-leucine)	Specific activity of protein (% ^{14}C-leucine added/mg. dry protein)
0	0.219
0	0.211
2.5	0.119
25	0.032
250	0.009

(c)

^{12}C-leucine added (µg.)	Relative specific activity of ^{14}C-leucine	* Specific activity of protein (% ^{14}C-leucine added/mg. dry protein)	Leucine incorporated (mµg/mg. protein)
0	1.000	0.096	1.81
0.19	0.909	0.089	1.86
1.90	0.500	0.060	2.26
19.0	0.091	0.010	2.13
190.0	0.010	0.005	-

* 0.19 µg. of ^{14}C-leucine added

than any achieved, at this time, using liver preparations.

I.4.3. Incorporation by microsome-'pH 5-enzyme' preparations: Incorporations into this preparation, under the conditions in Table 7, were measured after different times of incubation. Incorporation by this thyroid preparation was as high as that by the microsome-cell sap preparation. The preparations appeared to lose their activity after 40 min. at 37°.

I.4.4. Optimum ratio of 'pH 5-enzyme' to microsomes: Thyroid tissue (325 mg.) from four goitrous rats yielded 4.8 ml. of dilute centrifuged homogenate. From this, 0.75 ml. were removed for incubation. Microsomes and 'pH 5-enzyme' were isolated from the remainder. Finally, the microsomes were resuspended in 2.7 ml. III:IV (2.3:3, by volume) and the 'pH 5-enzyme' in 0.96 ml. of the same buffer. Three incubations were set up with different quantities of 'pH 5-enzyme' (Table 8).

By extrapolation of the final protein weights, against volume of 'pH 5-enzyme' solutions added, the quantity of microsomal protein was found to be 0.77 mg. per incubation, and the quantities of 'pH 5-enzyme' added were 1.5 mg., 2.5 mg., and 5.0 mg. A direct estimation of the concentration of ribosomal protein per ml. was not attempted because of the small quantity present. In total, 4.9 mg. of microsomal protein and 20.7 mg. of 'pH 5-enzyme' were isolated from 325 mg. of thyroid.

The specific activity of the purified protein rose with decreased quantity of 'pH 5-enzyme' although the total amount of ^{14}C-leucine incorporated fell. Two reported optimum ratios of enzyme to ribosomal protein are 4.4 mg. to 3.0 mg. (Zamecnik and Keller, 1957) and 3 mg. to 2 mg. (Korner, 1962) respectively. As the

microsomal fraction contains 68% protein and the ribosomal 46% protein, these ratios are equivalent to 1 mg. of 'pH 5-enzyme' with 1 mg. of microsomal fraction.

In most incorporations approximately 2 mg. of 'pH 5-enzyme' was added with as much microsomal material as possible up to 1-2 mg. of protein. To decrease the quantity of 'pH 5-enzyme' further than 2 mg. was not practical because small quantities of protein made the purification difficult and estimation of weight very inaccurate.

I.4.5. Addition of amino acid supplements:

I.4.5.1. Casein hydrolysate: During the isolation of the cell free system both the microsomes and 'pH 5-enzyme' underwent extensive washing. To supplement any depletion of amino acids, casein hydrolysate was added in different concentrations. $^{14}C(U)$-Leucine (0.5μC) at the specific activity available contained 0.19 μg. of the amino acid.

Incorporation into the supplemented incubations did not vary significantly (Table 9,a) but was lower than that into the unsupplemented control.

I.4.5.2. Complementary amino acids: The stable leucine in the casein hydrolysate may have decreased the specific activity of the $^{14}C(U)$-leucine 'pool' in the incubation sufficiently to mask the increased uptake of amino acids.

A mixture of amino acids in the proportions found in thyroglobulin (Derrien, Michel and Roche, 1949), without leucine, was added to the usual incorporation system. The mixture was added in molar proportions 2.5, 25 and 250 times the quantity of ^{14}C-leucine present.

Higher concentration of the supplement caused a marked decrease in the specific activity of the isolated protein (Table 9:b).

Table 10. Incorporation of ^{14}C-leucine by the microsomal-'pH 5-enzyme' preparation from rat thyroid with and without a source of ATP

Incubations under standard conditions (given in Table 7). Results from six preparations.

Specific activity of protein (%/mg.)		Inhibition (%)
Control	Without ATP	
0.219	0.211	-3.7
0.173	0.095	-41.4
0.151	0.095	
0.054	0.054	0
0.101	0.192	+50.0
0.107	0.120	
0.055	0.060	+9.1
0.054	0.046	-14.8

Table 11. Incorporation of ^{14}C-leucine by the microsomal-'pH 5-enzyme' preparation from rat thyroid with and without a source of ATP under nitrogen or 95% O_2 - 5% CO_2

Two preparations incubated under standard conditions (given in Table 7)

Gas phase during incubation	ATP-generating system	Specific activity of protein (% ^{14}C-leucine added/mg. dry protein)	Change with lack of ATP (%)
Air	+	0.130	
Nitrogen	+	0.028	
Nitrogen	+	0.031	
Nitrogen	-	0.046	+76
Nitrogen	-	0.056	
Air	+	0.062	
Air	+	0.063	
Oxygen	+	0.116	-48
Oxygen	-	0.063	

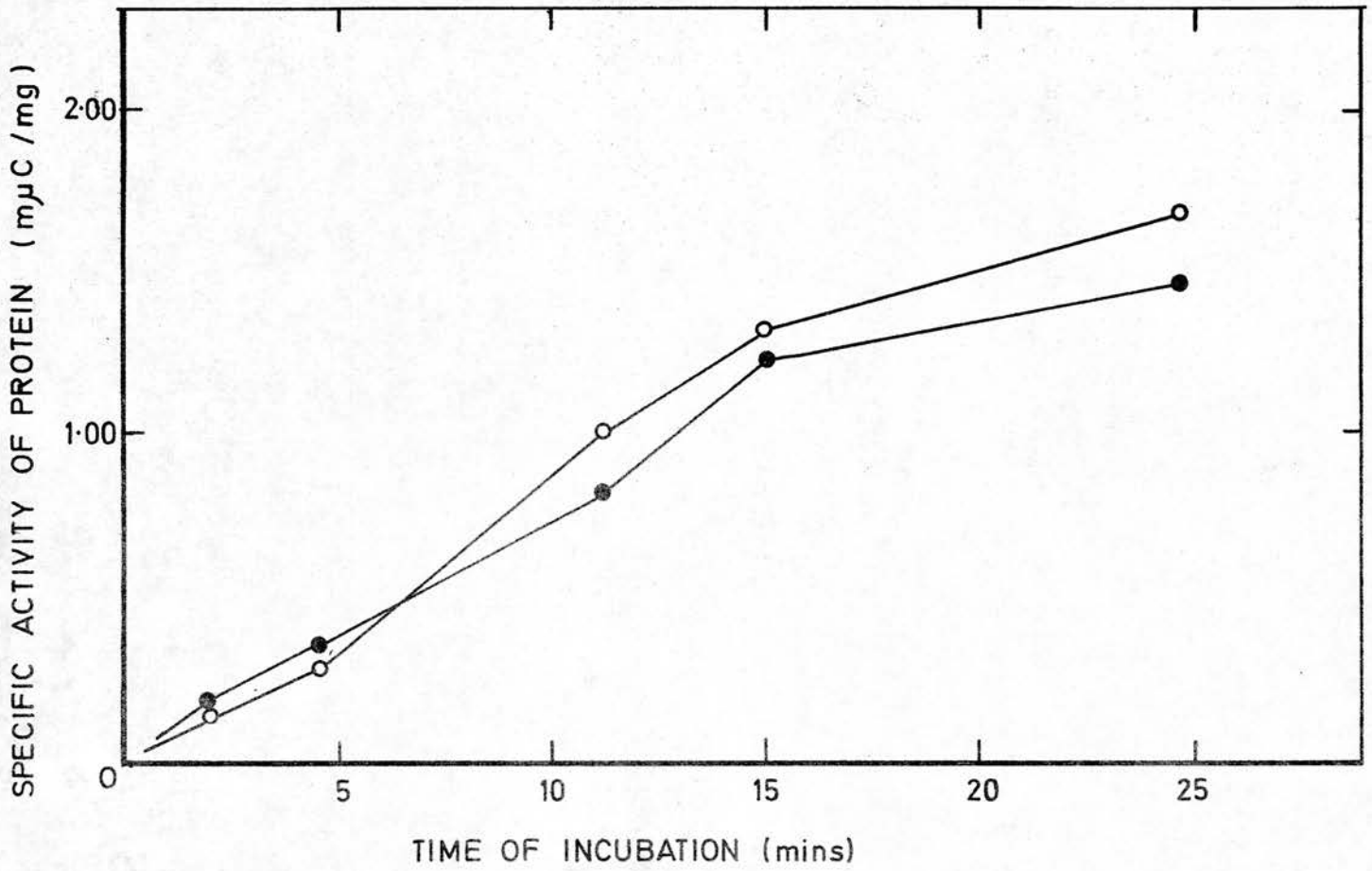


FIGURE 3 INCORPORATION OF ^{14}C-LEUCINE IN THE PRESENCE (●—●) AND IN THE ABSENCE (○—○) OF ATP AFTER DIFFERENT LENGTHS OF INCUBATION.

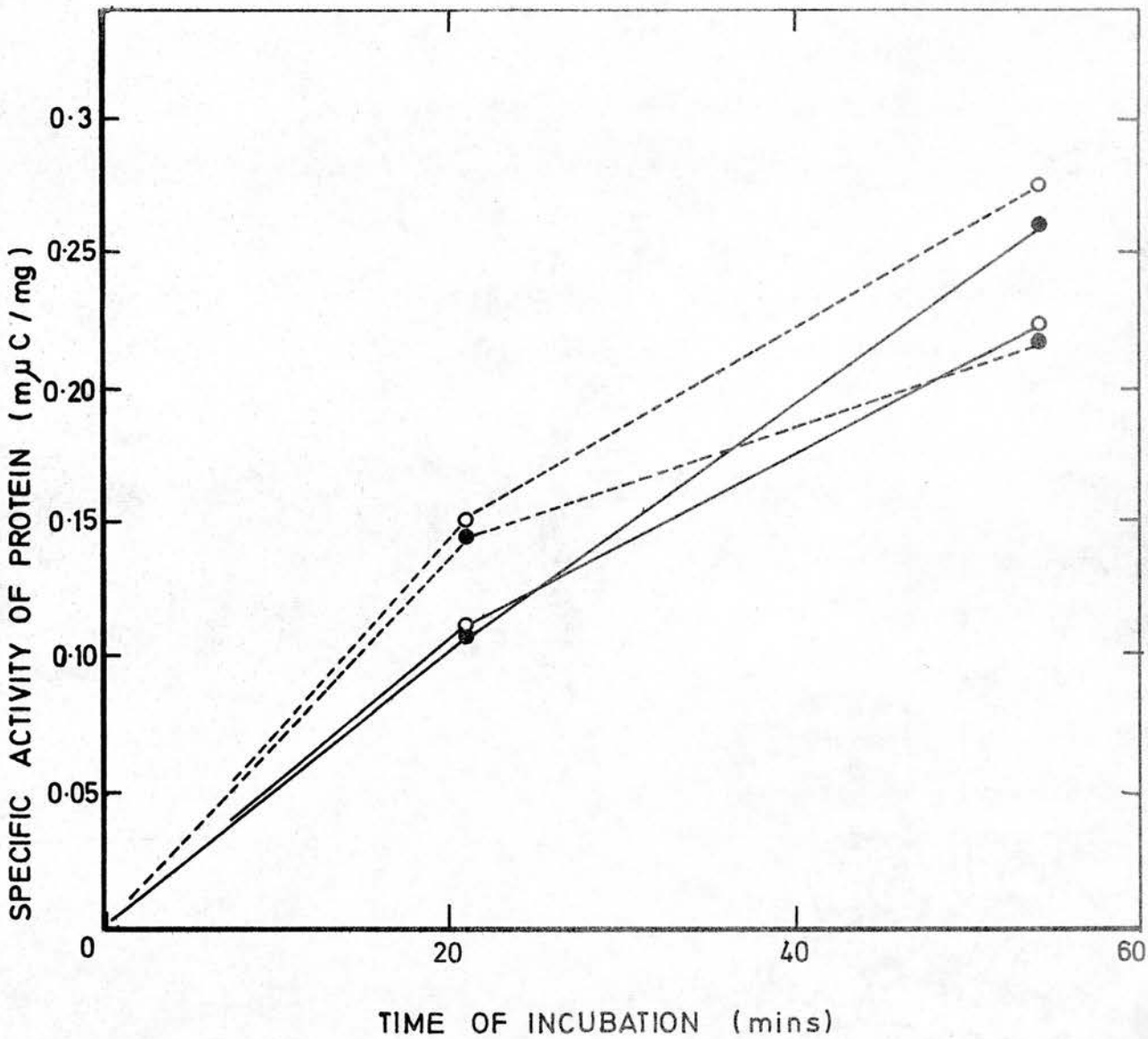


FIGURE 4 INCORPORATION OF ^{14}C-LEUCINE WITH (●—●) AND WITHOUT (○—○) ATP, IN OXYGEN (----) OR AIR (——) AFTER TWO LENGTHS OF INCUBATION.

I.4.5.3. Stable leucine: The specific activity of the 0.5 μC of ^{14}C-leucine added to each incubation was lowered by the addition of ^{12}C-leucine (Table 9:c). The specific activity of the purified protein decreased proportionately.

The total leucine incorporated at different concentrations of stable leucine remained fairly constant. The specific activity of the purified protein would have decreased less than proportionately on the addition of exogenous ^{12}C-leucine if the preparation had contained a high endogenous concentration of ^{12}C-leucine. That this does not happen indicates that the quantity of endogenous ^{12}C-leucine is, at most, of the same order as the added ^{14}C-leucine.

I.4.6. Energy dependence of the rat thyroid incorporating system:

I.4.6.1. In air: One of the accepted criteria of a protein synthesizing system is that it is energy dependent. Six experiments (Table 10) failed to show a consistent energy dependence.

It was thought possible that in the presence of an ATP-generating system the initial rate of incorporation might be increased, but that this activation was not maintained during 1 hr. incubations.

Over a range of times from 0-50 min. (Fig. 3) the uptake of ^{14}C-leucine in the unsupplemented system paralleled that in the energy supplemented system.

I.4.6.2. Under nitrogen: Although all incorporations were markedly decreased under nitrogen the uptake of ^{14}C-leucine remained higher in the absence of the ATP-generating system than in its presence (Table 11:a).

I.4.6.3. Under oxygen: Again no consistent differences were found in the rate of uptake of ^{14}C-leucine into protein in the presence or absence of the ATP-generating system under oxygen, (Fig. 4). In one experiment the oxygen stimulated incorporation (Table 11:b), but

Table 12. Localisation of incorporated ^{14}C-leucine label in the rat thyroid microsomal-'pH 5-enzyme' system

All fractions taken up in medium III : medium IV (2.3 : 3, by volume). Microsomal fraction (0.5 ml.), 'pH 5-enzyme' solution (0.25 ml.) and the ATP-generating system with 0.5 μC of ^{14}C-leucine (0.25 ml.) were incubated for 1 hr. The incorporation of label was halted by the addition of 10 ml. of 0.44 M sucrose containing a ten-fold excess of stable leucine. After centrifugation and washing the microsomal and soluble fractions were purified separately.

Fraction of incubation	Protein isolated (mg.)		Specific activity of protein (% ^{14}C-leucine added/mg. dry protein)		Distribution of ^{14}C-leucine (%)	
Total preparation	3.3		0.135		100	
Post-microsomal supernatant	1.4	1.3	0.0052	0.0040	2.8	3.6
Microsomal fraction	2.1	2.5	0.178	0.111	97.2	96.4

Table 13. Effect of goitrogen diet on the uptake of ^{14}C-leucine into the thyroid microsomal-'pH 5-enzyme' preparation

Goitrogen	MTU	MTU	PTU	PTU	PTU	MTU	PTU	PTU	TU	MTU	TU	All	TU
Days of diet	7	7	90	90	90	16	86	28	32	28	32	1-38	36
Incorporation into protein (%/mg.)	1.86	1.24	0.75	0.66	0.60	0.20	0.19	0.053	0.007	0.004	0.003	0.002	0.002

this was again not a consistent property of the system.

I.4.7. Effect of Mg^{++} concentration on incorporation into a centrifuged homogenate of rat thyroid: The homogenization and dilution media, III and IV, were made up with Mg^{++} at 0.5, 1.5, 4.0, and 6.0 mM. Under the usual conditions of incubation (Table 4, following p. 32) the level of incorporation did not alter significantly.

I.4.8. Localisation of label in a microsome-'pH 5-enzyme' system: After incubation for 1 hr. the incorporation was stopped by the addition of 10 ml. of 0.44M ice-cold sucrose containing a ten-fold excess of ^{12}C-leucine. The particulate fraction was sedimented at 125,000 *g* for 35 min., rinsed, and the washings and supernatant combined. The two fractions were purified separately and the specific activity of each found. Only 3.2% of the total activity was not found in the microsomal fraction (Table 12).

I.4.9. Effect of pre-treatment with goitrogen on isolated cell free systems: Centrifuged homogenate prepared from control rats and rats treated with TU showed no difference in activity.

In more active preparations there was no indication that the pre-treatment had any effect on the uptake of ^{14}C-leucine (Table 13). Any trend of this nature might, of course, be hidden in the large variation in levels of incorporation.

I.5. Chromatography of purified protein

A sample of purified protein containing 1.15 mμC/mg. of ^{14}C-leucine was chromatographed in butanol:2N-acetic acid (1:1, by volume). All the activity remained on the origin. Leucine in this system, has an R_f of 0.49-0.51.

I.6. Isolation of cell free systems from thyroids of other animals

I.6.1. Guinea pigs: Microsome-'pH 5-enzyme' systems were isolated

separately from the thyroids of three animals, of which one had had no goitrogen in its diet, one had been exposed to TU for 10 dy. and one to PTU for 14 dy. The thyroids from the three animals weighed 23 mg., 18 mg., 77 mg., and their respective amino acid incorporating abilities were .062% of ^{14}C-leucine added per mg., nil and nil. The possible advantage of larger amounts of tissue from guinea pigs was outweighed by their greater cost and the much greater difficulty of keeping the animals healthy on a goitrogen diet.

I.6.2. Oxen: These glands proved, even after slicing with a razor blade, or mincing in an MSE blender, to be too tough for homogenization by the usual method. Attempted homogenizations produced comparatively little microsomal material. Incorporation into these preparations was nil.

I.6.3. Hog: In one experiment using sucrose and medium II, and in a second experiment using media III and IV, incorporations of only 0.006% and 0.009% of the ^{14}C-leucine added per mg. of protein were produced by microsome-cell sap preparations.

I.7. Alternative methods of tissue disruption

To isolate a large amount of microsome-'pH 5-enzyme' system it was necessary to use an animal larger than the rat. With larger animals the thyroids became increasingly difficult to disrupt. A long and rough homogenization procedure tended to destroy the subcellular fractions soon after the cells were broken. A much gentler method of disrupting a good proportion of thyroid tissue would allow the isolation of comparatively large quantities of undamaged microsomal fraction which could be used to synthesize thyroglobulin of high specific activity.

Two methods which it was hoped would lead to gentle disruption were tried. Firstly, sonication of the tissue, and secondly

trypsinization to give free thyroid cells which could then be homogenized under very mild conditions or lysed in a hypotonic medium.

I.7.1. Sonication as a means of disrupting thyroid tissue: Sonication of minced thyroid tissue had to be a compromise between long, hard sonication, necessary for complete disruption of the tissue, and the necessity to keep the temperature low and the already freed sub-cellular fractions relatively undamaged.

The MSE Ultrasonicator used could be fitted with a $\frac{3}{4}$" or $\frac{1}{4}$" diameter probe. Of these, the former was discarded as it produced a large heating effect and its power was not needed for the small volumes used.

The $\frac{1}{4}$" diameter probe at full power rapidly caused the medium to froth, denaturing freed protein. Even at less than maximum power the probe liberated 60-100 calories per min.

This heating effect could be overcome only by immersing the sonication vessel in an ice and salt mixture with a temperature approaching -10°. If this mixture was too cold the medium froze, otherwise, especially with longer sonication, the temperature rose to 20° or 30°. Maintenance of a stable temperature between 0° and 3° was achieved by sonicating the tissue in a small nickel crucible. This, being flat-bottomed, had a larger surface area than the thick-walled glass tubes which had been used up till then, and the thin metal walls conducted heat rapidly into the surrounding ice and salt mixture.

With the ice and salt mixture at -5°, 30 sec. spells of sonication were alternated with 30 sec. spells without sonication to allow the medium to cool. Using this method, and taking the temperature of the medium after each burst of sonication, the maximum temperature could be kept at 3.5° for an indefinite period.

If the temperature began to rise the length of the cooling spells were increased or the length of sonication decreased.

Using the sonicator in this way 300 mg. of minced rat liver in 3 ml. medium III was totally disrupted in less than 30 sec. with no significant rise in temperature.

Very thin slices pared from deep-frozen human thyroid were used as a suitable test material to study the efficiency of the sonicator in disrupting tougher tissues. Little disruption of the slices occurred within 15 min. and 30-60 min. of sonication were required before the slices lost their structure and the supernatant became cloudy.

The degree of sonication was measured by the loss in weight of the tissue present originally or by the quantity of material liberated.

Slices cut from the deep-frozen tissue were suspended in ice-cold sonication medium, shaken and sedimented at 1,000 g. The tubes were dried with tissue paper and weighed. After sonication the material was again sedimented and the tube dried and reweighed. The difference in weight was taken to be the quantity of material which had been dispersed by the sonication procedure.

From four experiments the percentage sonication rose to over 30% in about 1 min. and remained between 35% and 45% for over 10 min. At 15-30 min. the sonication rose to nearly 60%.

The quantity of protein in the supernatant after the sonicated tissue had been sedimented at 15,000 g for 10 min. was estimated by its optical density at 280 mμ taking an $E^{1\%}_{280}$, 1 cm. as 10. (Salvatore, Salvatore, Cahnmann and Robbins, 1964). By this method approximately 100 mg. samples of thyroid slices released 14.0, 17.0, and 15.1% of their original weight as soluble protein in 2.5, 5

and 7.5 min.

Similarly 1.00 g. of slices, sonicated for 15 min., released 141 mg. of protein into the post-mitochondrial supernatant. This supernatant was centrifuged at 125,000 g to sediment the microsomes. The post-microsomal supernatant contained 116 mg. of protein and the microsomes 15.3 mg., making a total of 131 mg. as against the original 141 mg. The difference was probably within experimental error. The percentage yield of microsomes, 1.5%, and of cell sap, 13%, are comparable with the yields from rat thyroid after homogenization (p.35).

These results were taken as an indication that sonication under the above conditions is capable of disrupting thyroid tissue sufficiently to release considerable quantities of sub-cellular material.

Microsome-'pH 5-enzyme' preparations were isolated from finely minced rat thyroids after sonication from 5 up to 30 min. in medium III. In no case was there any incorporation.

Apparently the sub-cellular particles had been damaged during or after their release from the disrupted cells.

I.7.2. Trypsinization as a means of dispersing thyroid cells: Two types of trypsinization were attempted. One method consisted of batch incubation of rat thyroid with trypsin, and the other, on a larger scale, of continuous incubation of pig thyroids.

The former method followed the procedure of Pulvertaft, Davies, Weiss and Wilkinson (1959) and Dulbecco and Vogt (1954). The trypsinization medium was a phosphate buffered saline (PBS) made up in three parts, autoclaved, cooled and mixed as described by Barzelatto, Murray and Stanbury (1962). To this solution was added 10,000 i.u. of streptomycin sulphate and 10,000 i.u. sodium

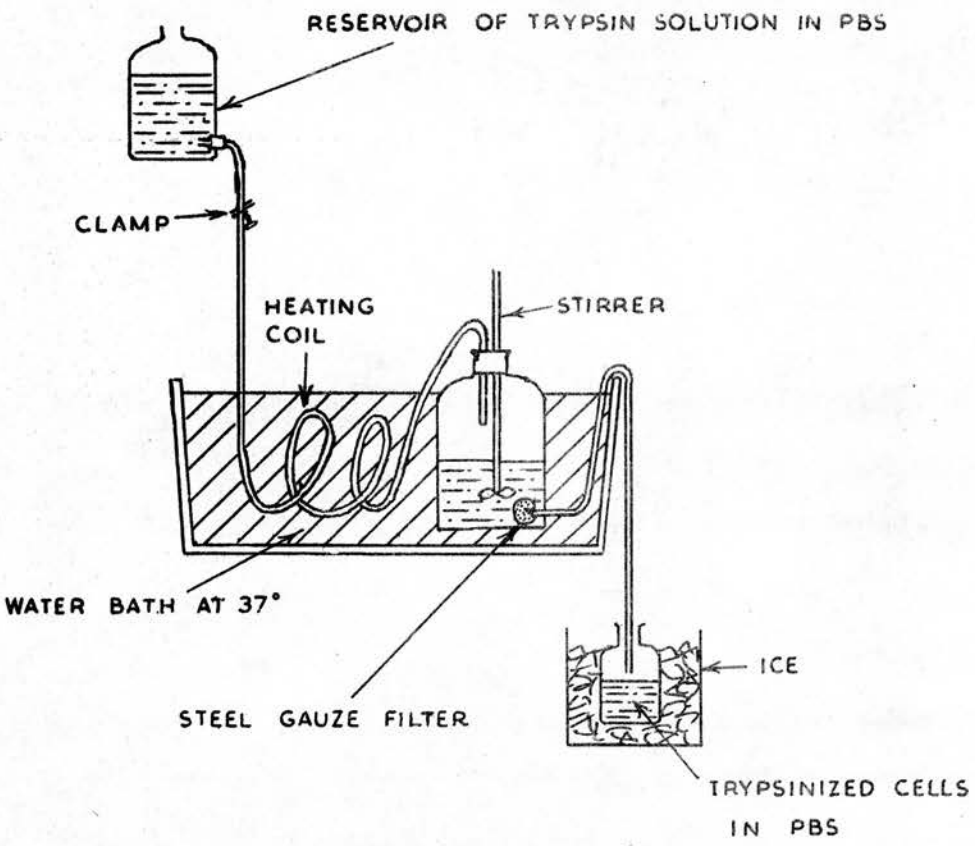


FIGURE 5 APPARATUS FOR THE CONTINUOUS TRYPSINIZATION OF RAT OR SHEEP THYROID TISSUE.

benzylpenicillin per 100 ml. BDH Trypsin, to 0.25%, was added to the PBS. This solution was stored overnight at 4°, passed through a Seitz filter and stored at -20°.

Rat thyroid was finely minced with a scalpel and added to 20 volumes of the trypsin solution in PBS. The mixture was incubated at 37° for 30 min., shaken vigorously by hand, allowed to settle and the supernatant decanted.

A further 15 volumes of PBS-trypsin solution was added and the incubation continued at 37° with shaking by hand every 15 min. By microscopic examination of samples of the supernatant, free cells first appeared at 2 hr. Incubation was continued for up to 20 hr. when free cells were still visible.

To separate the cells, some of which were in loose clumps, the medium was passed through stainless steel gauze, 160 mesh, and the filtrate cooled in ice and centrifuged at 600 g for 10 min. A small pellet of cells sedimented and none was found in the supernatant. After washing, the cells were resuspended in medium IIIc and homogenized with one stroke of a Teflon-glass homogenizer.

Incorporation into the microsome-'pH 5-enzyme' system was up to 0.60% of the ^{14}C-leucine added per mg., but the system again was not energy dependent. Only a very small quantity of 'pH 5-enzyme' was isolated and the total protein yield from 408 mg. of thyroid was 3.9 mg.

The low yield of a reasonably active incorporation system led to the setting up of a continuous trypsinization procedure after the method of Tong, Kerkof and Chaikoff (1962). With the continuous system it was hoped to use larger quantities of pig thyroid with a concomitant increase in the yield of epithelial cells.

The continuous trypsinization apparatus is shown in Fig. 5. The sterile PBS-trypsin solution was warmed to 37° before entering the

flask by passing slowly from the reservoir through a polythene tube immersed in the water bath.

The flow rate was controlled so that the volume in the flask was constant. Stirring was continuous but at a low speed to avoid frothing. The supernatant was slowly siphoned off through a small piece of 160 mesh steel gauze into a reservoir in ice. Approximately 50 ml. were run through per hr. Drops of the medium leaving the flask were examined regularly for the presence of free cells.

Pig thyroid (10 g.) finely minced with razor blades, was added to 50 ml. of trypsin solution. The mixture was shaken and added to the flask. After 2 hr. cells were noted in the filtrate. The first 100 ml. to come over were discarded. After 4 hr. of incubation no more free cells were seen in the effluent from the flask.

The quantity of free cells, as judged microscopically, was much smaller than had previously been removed from rat thyroid. Sedimentation of the cells confirmed this. Later attempts did not show an increased yield of cells.

The minced tissue tended to form long strings wound round the stirrer, perhaps preventing the trypsin from acting fully on the tissue.

Neither of the alternative methods of tissue disruption had yielded a more active cell free protein synthesizing system. It was felt that thyroid tissue posed special problems in as much as the mechanical strength of the tissue required vigorous methods of disruption and these in turn were sufficient to inactivate much of the protein synthesizing system.

Chapter II

INCORPORATION IN VITRO OF ^{14}C-AMINO ACIDS BY SURVIVING THYROID PREPARATIONS

The previous chapter reports the difficulty that was experienced in the isolation of a cell free system for the incorporation of labelled amino acids into thyroglobulin. The major loss of activity was thought to occur during homogenization. However, surviving thyroid preparations, which had experienced the minimum of disruption, had been shown (Ragupathy, Abraham, Kerkof and Chaikoff, 1964) to incorporate amino acids actively, and to iodinate thyroglobulin in a way very similar to the *in vivo* situation.

The aim at this stage of the work was two-fold: firstly, by changes in the incubation conditions, to increase the incorporation of ^{14}C-amino acids into the surviving thyroid preparations, and, secondly, to isolate partially purified thyroglobulin from these preparations. Larger incubations and higher concentrations of ^{14}C-amino acids were expected to increase incorporation and it was hoped, as thyroglobulin is the major protein synthesized, that this would also prove to be the most highly labelled protein. The initial experiments were to be carried out with ^{14}C-leucine as the source of label, principally because this is in high concentration in thyroglobulin, and leucine undergoes very few side reactions (Ragupathy *et al.*, 1964). However, in order to label iodotyrosyl residues in thyroglobulin with carbon-14, it was necessary to use ^{14}C-tyrosine as the source of label. Ultimately the labelled thyroglobulin was to be partially hydrolyzed and these iodotyrosyl

residues separated as peptides. The incorporation of several ^{14}C-amino acids simultaneously with the ^{14}C-tyrosine was expected to augment the specific activity of these peptides. Finally, thyroglobulin labelled with ^{131}iodine was to be prepared by adding ^{131}iodide to the incubation medium of the surviving thyroid preparations.

II.1. Methods

II.1.1. Isolation of thyroid-trachea preparation: Ether-anaethetized male Wistar rats had a mid-line incision made in the ventral aspect of the neck. The salivary glands and lymph nodes were moved. The sternomastoids were eased aside and the sternohyoid cut at the point of attachment to the hyoid and removed. This exposed the trachea with the attached thyroid partially covered by the sternothyroids which were removed. The trachea was cut about 2 mm. posterior to the thyroid and freed from the underlying oesophagus. Section of the larynx, well clear of the anterior end of the thyroid, completed the removal of the preparation.

II.1.2. Incubation conditions: The medium used for incubation was Krebs Ringer Phosphate Bicarbonate-III (KRPB-III) (Umbreit, Burris and Stauffer, 1964) which had added to it 600 mg./100 ml. of D-glucose. Unless otherwise stated the incubation volume was 0.21 ml. containing 0.1 μC of ^{14}C-leucine. The incubation medium and the ^{14}C-leucine were pipetted into small conical centrifuge tubes and preheated to 37° in a water bath. Immediately after dissection the thyroid-trachea preparations were added to the medium, the tubes flushed with O_2:CO_2 (95%:5%) and stoppered. Incubation was continued at 37° for different periods of time between 1 and 24 hr.

II.1.3. Isolation of protein and sub-cellular fractions after incubation: After incubation the tubes were cooled in ice and the

Table 14. Incorporation of ^{14}C-leucine by rat thyroid-trachea preparations

(a) Incubation in 2.01 ml. of KRPB-III with 0.1 μC of ^{14}C-leucine for 1 hr.

Protein fraction	Protein weight (mg.)	Specific activities of protein (mμC ^{14}C-leucine /mg.)	(% ^{14}C-leucine added /mg.)
Sediment at 15,000g	8.4	0.158	0.158
	7.4	0.254	0.254
	7.6	0.088	0.088
	6.7	0.120	0.120
Cell sap	15.4	0.096	0.096
	14.1	0.131	0.131
	10.2	0.059	0.059
	7.9	0.159	0.159
		(mμC)*	(%) *
Microsomal fraction	< 1.0*	0.115	0.115
	-	0.138	0.138
	-	0.046	0.046
	-	0.208	0.208

(b,i) Incubation in 0.31 ml. of KRPB-III (100 mg. of glucose / 100 ml.) with 0.1 μC ^{14}C-leucine for 3 hr.

Sediment at 15,000g	4.6	1.62
	3.6	1.18
Cell sap	2.8	2.72
	3.5	2.44
	-	(%)*
Microsomal fraction	-	1.29
	-	0.88

(b,ii) Incubation in 0.21 ml. of KRPB-III (600 mg. of glucose / 100 ml.) with 0.1 μC ^{14}C-leucine for 4.75 hr.

Sediment at 15,000g	6.5	0.72
	5.9	0.62
	5.9	0.65
	6.4	0.66
Supernatant at 15,000g	7.8	0.47
	5.5	0.68
	5.9	0.76
	7.3	0.50

* Quantity of protein was not sufficient to allow determination of its weight.

thyroids were dissected from the trachea on an upturned petri dish sunk in crushed ice. They were then weighed, homogenized in a small all-glass homogenizer and the sub-cellular fractions were isolated as indicated on pp. 26-7.

To extract thyroglobulin, the thyroids, freed from surrounding tissue and washed, were frozen on an upturned petri dish resting on crushed solid CO_2. The hard glands were then sliced finely with a razor blade. The slices were gently shaken in the cold with 0.9% NaCl overnight and the solid material was centrifuged off at 15,000 g. The supernatant from this, or the cell sap from homogenized glands, was sometimes fractionated with ammonium sulphate by the method of Derrien, Michel and Roche (1948) as outlined in the section on purification of thyroglobulin (Section III.1.). All protein and sub-cellular fractions were isolated, purified and assayed for radioactivity as detailed in Sections I.2.4. and I.2.8.

II.2. Results

II.2.1. Incorporation into rat thyroid-trachea preparations: Table 14 lists the levels of incorporation of ^{14}C-amino acids into the thyroid-trachea preparations under different conditions of incubation.

Initially the preparations were incubated in 2.01 ml. of KRPB-III with 0.1 μC of ^{14}C-leucine for 1 hr. (Table 14:a). After this length of incubation, the thyroids had not visibly deteriorated and it was thought that longer incubation might result in a greater incorporation. Glucose was added (to a level of 100 mg./100 ml. medium) in case the thyroids had utilised much of their endogenous substrates. The concentration of ^{14}C-leucine was increased by decreasing the incubation medium to 0.31 ml. Incubations were carried out in 10 ml. conical centrifuge tubes to ensure that the

Table 14. Incorporation of ^{14}C-leucine by rat thyroid-trachea preparation (cont.)

Protein fraction	Protein weight (mg.)	Specific activity of protein (% ^{14}C-amino acid added/mg. dry protein)
(b,iii) Incubation in 0.21 ml. of KRPB-III (600 mg. glucose/100 ml.) with 0.1 μC ^{14}C-leucine for 2 hr.		
Sediment at 15,000g	5.0	0.390
	3.2	0.425
Supernatant at 15,000g	3.4	0.810
	4.5	0.714
(c) Incubation in 0.21 ml. of rat serum (500 mg. glucose/100 ml.) with 0.33 μC ^{14}C-leucine for 3 hr.		
Sediment at 15,000g	5.6	0.46
	5.3	0.49
Supernatant at 15,000g	4.0	0.29
	3.1	0.42
(d) Incubation in 0.22 ml. of KRB (no glucose) with 0.56 μC ^{14}C-leucine for 3 hr.		
Sediment at 15,000g	5.6	0.29
	4.2	0.43
Supernatant at 15,000g	7.6	0.22
	5.0	0.30
Incubation medium	3.1	0.77
	3.0	0.51
(e) Incubation in 0.22 ml. of KRPB-III (100 mg. glucose/100 ml.) with 0.1 μC ^{14}C-tyrosine for 7 hr.		
Sediment at 600g	5.2	0.38
	3.7	0.66
Sediment at 10,000g	1.5	0.62
	0.7	0.31
Microsomal fraction	0.4	1.25
	0.4	0.96
Cell sap	5.1	0.32
	3.4	0.97

Table 15. Dependence of incorporation of ^{14}C-leucine by thyroid-trachea preparations on the leucine concentration

(a) Incubation in 0.22 ml. of KRPB-III (600 mg. glucose/100 ml.) for 1 hr.

Protein fraction	^{14}C-leucine added (μC)	Incorporation (% ^{14}C-leucine added)	Protein isolated (mg.)	Specific activity of protein (mμC ^{14}C-leucine /mg.)
Sediment at 15,000g	0.05	4.76	15.6	0.16
	0.10	3.86	6.3	0.61
	0.20	3.48	9.4	0.71
	0.40	3.25	6.0	2.03
Supernatant at 15,000g	0.05	6.09	27.9	0.12
	0.10	6.43	16.5	0.39
	0.20	6.45	15.6	0.79
	0.40	6.72	12.0	2.36
Whole thyroid, i.e. sum of above	0.05	10.85	43.5	0.12
	0.10	10.26	27.8	0.37
	0.20	9,93	25.0	0.76
	0.40	10.87	18.0	2.25

(b) Incubation in 0.22 ml. of KRPB-III (600 mg. glucose/100 ml.) with 0.2 μC ^{14}C-leucine (2.33 mμmole) for 1 hr.

Protein fraction	Leucine added (mμmole)	Incorporation (% ^{14}C-leucine added)	Protein isolated (mg.)	Leucine incorporated (mμmole/mg. protein)
Sediment at 15,000g	1.25	1.79	5.5	0.012
	2.83	1.70	6.0	0.015
	9.16	3.10	6.0	0.059
	17.09	3.26	10.6	0.060
	32.93	1.04	4.8	0.076
	64.61	1.75	4.3	0.172
Supernatant at 15,000g	1.25	4.75	17.6	0.010
	2.83	4.00	16.2	0.013
	9.16	5.07	11.7	0.050
	17.09	6.60	26.7	0.048
	32.93	2.86	9.9	0.102
	64.61	3.61	9.4	0.256
Whole thyroid, i.e. sum of above	1.25	6.54	23.1	0.022
	2.83	5.70	22.2	0.028
	9.16	8.16	17.7	0.109
	17.09	9.86	37.3	0.108
	32.93	3.80	14.7	0.178
	64.61	5.36	13.7	0.428

preparations remained covered in medium. These measures resulted in increased incorporation (Table 14:b,i).

Further alterations in glucose concentration, volume of incubation medium, and length of incubation did not alter the incorporations consistently (Table 14:b,ii & iii).

II.2.2. Incorporation with other incubation media: Rat blood, removed by heart puncture, was allowed to clot and the serum separated from the thrombus by centrifugation. The serum was supplemented with 500 mg./100 ml. of D-glucose and 'gassed' by bubbling O_2:CO_2 (95%:5%) through it.

Kerbs Ringer Bicarbonate (Umbreit, Burris and Stauffer, 1964) was also substituted for the usual KRPB-III.

In neither case was the incorporation increased above previous levels (Table 14:c and d, respectively).

Both KRB and rat serum in the small quantities used were susceptible to loss of CO_2 with a consequent rise in pH. In addition rat serum took some time to prepare, was available only in limited quantities and was subject to unknown variations in composition. All later incubations of thyroid-trachea preparations used KRPB-III.

II.2.3. Incorporation with increased concentrations of ^{14}C-leucine: Increasing the concentration of ^{14}C-leucine in the incubation medium caused more of the amino acid to be taken up by the thyroid and incorporated into cell proteins.

Quantities of ^{14}C-leucine ranging from 0.05 μC to 0.4 μC were added to thyroid-trachea incubations. The specific activities of fractions, separated by centrifugation at 15,000 g for 20 min. (Table 15:a) were proportional to the concentration of ^{14}C-leucine in the incubation medium. The percentage of the added ^{14}C-leucine

Table 15. Dependence of incorporation of ^{14}C-leucine by thyroid-trachea preparations on the leucine concentration

(cont.)

(c) Incubations in 0.22 ml. of KRPB-III (600 mg. glucose/100 ml.) with 0.40 μC ^{14}C-leucine (4.66 mμmoles) for 3.5 hr.

Protein fraction	Leucine added (mμmoles)	Incorporation (% ^{14}C-leucine added)	Protein isolated (mg.)	Leucine incorporated (mμmoles/mg. protein)
Sediment at 15,000g	2.0	0.775	4.3	0.011
	29.2	0.405	2.7	0.050
	58.2	0.480	4.9	0.061
	131.0	0.335	5.3	0.085
	196.0	0.323	4.6	0.141
Supernatant at 15,000g	2.0	0.737	4.3	0.011
	29.2	0.542	3.1	0.059
	58.2	0.395	4.4	0.056
	131.0	0.339	3.7	0.126
	196.0	0.400	3.9	0.206
Whole thyroid, i.e. sum of above	2.0	1.512	8.6	0.022
	29.2	0.947	5.8	0.109
	58.2	0.875	9.3	0.117
	131.0	0.674	9.0	0.211
	196.0	0.723	8.5	0.347

incorporated per gland was nearly constant.

This effect was investigated further by diluting 0.1μC of ^{14}C-leucine with increasing amounts of ^{12}C-leucine. Once more incorporation paralleled the concentration of leucine (Table 15:b).

In a third experiment of this type ^{12}C-leucine was again used to dilute the ^{14}C-leucine. Up to 100 times the standard concentration of cold leucine was added to give an approximately proportional rise in incorporation (Table 15:c).

Addition of complementary amino acids gave no indication of an increased level of incorporation.

II.2.4. Incorporation of ^{14}C-tyrosine (Table 14:e): ^{14}C-Tyrosine (0.1μC) was substituted for the ^{14}C-leucine. The lower incorporation may reflect the lower tyrosine content of thyroglobulin - 125 tyrosyl residues against 502 leucyl residues per molecule.

II.2.5. Chemical binding of incorporated ^{14}C-leucine in thyroid protein: Although the procedure for the removal of non-incorporated ^{14}C-amino acids was thought to be very effective, thyroid protein was enzymically hydrolysed to demonstrate the release of ^{14}C-leucine.

Two thyroid-trachea preparations were incubated under standard conditions for 5 hr. After washing and dissection, the thyroids were homogenized in 0.04M Tris-HCl buffer containing 0.11M NaCl, 0.04% TU and 0.004M Mn^{++} (Tong and Chaikoff, 1958). Pancreatin was added to half the homogenate and the remainder was purified and counted.

After 48 hr. the pancreatin hydrolysate was centrifuged and samples of the supernatant and the unhydrolysed purified protein were chromatographed in butanol:acetic acid (BA) and butanol:dioxane:ammonia (BDA) overnight (Section V.1.5). Counting of the strips on the BTL Chromatogram Scanner showed that all the activity in the

Table 16. Incorporation of ^{14}C-leucine into ammonium sulphate fractions of rat thyroidal protein by thyroid-trachea preparations

Preparations incubated in 0.21 ml. of KRPB-III (600 mg. glucose/100 ml.) containing 0.1 μC of ^{14}C-leucine for 4.75 hr.

Time of incubation (hr.)	Specific activity of protein fractions (% ^{14}C-leucine added/mg. of protein)			
	Sediment at 15,000g	Protein soluble in 42% $(NH_4)_2SO_4$, (a) & (c), and 45% (b)	Protein insoluble in 37% $(NH_4)_2SO_4$, (a) & (c), and 35% (b)	'thyroglobulin' i.e. remainder
(a) 5	-	0.100	0.20	0.032
(b) 14	2.84	0.26	-	0.98
	1.91	0.40	-	0.87
(c) 0.5	0.25	0.25	1.37	0.20
1	0.33	0.44	2.82	0.35
2	0.50	1.08	5.61	0.94
3	0.82	0.89	4.18	0.87
4	1.43	1.74	5.34	3.49
5	0.73	1.26	7.38	1.66
12	1.82	0.52	-	-
24	1.16	-	-	-

unhydrolysed protein remained on the origin, whereas all the activity from the hydrolysed protein ran with an R_f of 0.51-0.52 in BA and 0.33-0.35 in BDA. These values correspond to the R_f values of leucine in BA and BDA.

The chromatography indicates that the amino acid is chemically incorporated into the protein and can be released as the free amino acid only by total enzymic hydrolysis.

II.2.6. Incorporation into partially purified thyroglobulin: In earlier experiments improvement of the conditions for incorporation had produced increasingly labelled protein preparations. If, however, thyroglobulin was being synthesized more rapidly than other thyroidal proteins isolation of thyroglobulin from the crude thyroid protein preparation would give a more highly labelled product.

The classical method of ammonium sulphate fractionation (Section III.1.) was used to separate thyroglobulin from other thyroidal proteins. In some cases, (cf. particularly Table 17:d), partially purified thyroglobulin had an increased specific activity.

The small quantities of starting material led to difficulties in fractionation. Each g. of thyroid yields 50 mg. of purified thyroglobulin (Derrien, Michel and Roche, 1948). Approximately 1 mg. quantities of partially purified thyroglobulin were isolated from thyroids of non-goitrous rats. Working with this amount of material resulted in inaccuracies in fractionation. In some experiments thyroglobulin had to be estimated spectrophotometrically.

The results in Table 16:a were obtained after fractionation of a saline extract from the thyroids of four non-goitrous control rats. The low specific activity of the 'thyroglobulin' fraction may have been caused by the time lag between uptake of ^{14}C-leucine into the gland and the appearance of ^{14}C-protein in the lumen of the

Table 17. Effect of PTU in diet on the incorporation of ^{14}C-leucine by rat thyroid-trachea preparations

Incubations were carried out in 0.22 ml. of KRPB-III (600 mg. glucose /ml.) containing 0.2 μC ^{14}C-leucine for 1 hr. (a) and (c), 2 hr. (b), and 2 hr. 20 min. (d).

Experiment and time of incubation (hr.)	PTU in diet	Incorporation into fractions isolated (%/mg.)	
		Sediment at 15,000g	Supernatant
(a) 1	None	0.221	0.218
	4 wk. until sacrifice	0.092	0.118
(b) 2	None	0.65	0.88
	4 wk. until sacrifice	0.27	0.36
		0.28	0.19
	Removed 32 dy. before sacrifice	0.47	0.71
		0.39	0.75
(c) 1	None	0.87	1.31
		0.68	1.31
	None - low iodine diet	0.25	0.35
		0.53	0.45
	Removed 96 dy. before sacrifice	0.43	0.60
		0.45	0.39
	Removed 32 dy. before sacrifice	0.18	0.25
		0.15	0.22

(d)

PTU in diet	Wet wt. of thyroids (mg.)	Incorporation into fractions isolated (%/mg.) Sediment at 15,000g	Protein soluble in 45% A.S.+	Protein insoluble in 35% A.S.+	Thyroglobulin
None	18.1	0.75	-	1.26	5.60
	25.5	0.96	-	1.74	6.98
	18.3	0.58	-	2.46	7.18
	20.0	0.45	-	0.67	2.86
	20.5 ± 3.0^x	0.68 ± 0.19^x	-	1.54 ± 0.66^x	$5.66 \pm 1.02^{x*}$
2 mth. & removed 52 dy. before sacrifice	85.7	0.47	0.68	0.20	1.00
	80.8	0.51	0.81	0.38	0.94
	92.3	0.35	-	0.25	0.90
	86.6	0.24	0.47	0.21	0.91
	86.4 ± 4.1^x	0.40 ± 0.10^x	0.65 ± 0.14^x	0.26 ± 0.046^x	$0.94 \pm 0.038^{x*}$

^{x} Average $\pm$ standard deviation

* $0.001 < P < 0.005$

$^+$ $(NH_4)_2SO_4$

follicles.

This was indicated by the results in (b) and (c) where the cell sap, isolated after homogenization, was fractionated. In (b), after 14 hr. incubation, 'thyroglobulin' was the most highly labelled soluble fraction. The average yield of 'thyroglobulin' in experiment (c) was only 0.7 mg. from 40 mg. of tissue, demonstrating a loss of this protein during fractionation.

Ammonium sulphate fractionation of such an amount of protein was not practicable and attention was turned to production of larger quantities of starting material.

II.2.7. Effect of goitrogen pre-treatment on incorporation of ^{14}C-leucine: In an attempt to increase the quantity of thyroglobulin available for fractionation thyroid-trachea preparations from goitrous rats were used. Some of the low incorporations by these preparations were thought to be related to the pre-treatment with thiouracils. As this was contrary to the findings with the in vitro cell free synthesizing system (section I 4.9, p.38), and, as any effect of this kind would have to be known before adoption of this method of preparation of thyroglobulin, a study was made of the goitrogen effect.

PTU treatment was found to decrease the level of incorporation of ^{14}C-leucine into thyroid protein (Table 17). This was true also of goitrous tissue from rats on a low iodine stock diet. Thyroglobulin isolated from goitrous glands contained much less label than did the protein isolated from non-goitrous glands (Table 17:d).

Since hyperplastic glands contain less stored thyroglobulin the decrease in specific activity was caused by a loss of activity of the protein synthesis system. The greater quantity of microsomal material isolated from the goitrous glands may have masked this effect in the in vitro (cell-free) preparations.

II.3. Methods for incorporation of ^{14}C-amino acids into thyroid slices

II.3.1. Incubation medium: Krebs Ringer Bicarbonate (KRB) was adopted as the medium for incubation of thyroid slices. This buffer has been shown to be better for tissue slices (Umbreit et al, 1964) especially if 'gassed' with O_2 containing CO_2. No glucose or Krebs cycle intermediates were added to the medium as Ragupathy et al., (1964) had found these did not increase incorporation.

II.3.2. Preparation of slices: Thyroid tissue, usually from sheep or lambs, was obtained from a local slaughterhouse. The tissue was kept cold, in ice, at all times and dissection was carried out in a cold room. The glands were trimmed and sliced on filter paper soaked in KRB on a chilled glass plate. Each was sliced with a new safety razor blade.

Slices, prepared as above, were weighed and their areas were measured. The average thickness found to be 0.25-0.30 mm. This ensured that all cells in the slices had an adequate oxygen supply (Umbreit et al., 1964).

II.3.3. Incubation conditions: Newly cut slices from different glands were pooled and kept in cold KRB until sufficient tissue had been cut. Weighing the KRB before and after gave the weight of tissue prepared. Gentle swirling of the slices in the medium removed any unwanted material, such as wool, and washed much of the blood from the tissue. Fortunately, the sheep were bled immediately after death so that the thyroid contained little blood. Washing in KRB also removed considerable quantities of protein from cut cells and follicles.

The slices were removed from the KRB, roughly blotted with filter paper and added to 10 volumes of KRB, containing the ^{14}C-amino

acid, in an 150 ml. conical flask or a test tube, depending on the weight of tissue.

The final incubation medium was made up by adding 1 volume of ^{14}C-amino acid to 10 volumes of a saline containing all the salts of KRB in concentrations 1.1 times those in KRB. The slices and medium were kept cold until the flask had been sealed with a Suba-seal and flushed with O_2-CO_2. After a 4 hr. period of incubation at 37° the flask was flushed again.

At the end of the incubation the flask was chilled in ice and the contents were thoroughly homogenized in a chilled glass homogenizer.

In some experiments the slices were removed, with washing, from the incubation medium which contained considerable quantities of labelled protein.

II.3.4. Isolation of sub-cellular fractions and soluble proteins: Sub-cellular fractions were isolated as detailed in Sections I.2.3. and I.2.4. and assayed for radioactivity. The cell sap, and incubation medium, if separated, were initially assayed by the usual method (Section I.2.8.) but in later experiments these soluble fractions were dialysed against three changes of 2 l. of 0.1N NH_4OH (pH 11). The dialysis residue was shell-frozen and freeze-dried.

This treatment removed all contaminating ^{14}C-amino acid. Small samples of the dry protein were assayed for radioactivity, which was expressed as μC/mg., not, as previously, percentage of amino acid incorporated per mg.

II.4. Results of incorporation of ^{14}C-amino acids into sheep thyroid slice tissue

II.4.1. Incorporation into fractions after different times of incubation:

Table 18. Incorporation of ^{14}C-leucine by sheep thyroid slices

Sheep thyroid slices (100 mg.) were incubated with 1.67 μC of ^{14}C-leucine in 1.1 ml. of KRB for 1, 2 and 3 hr., removed from the incubation medium with washing, homogenized in KRB and fractionated by centrifugation at 1,000g and 10,000g for 20 min.

Protein fraction	Specific activities of protein isolated (mμC/mg. protein)		
Time of incubation (hr.) -	1	2	3
Whole thyroid	3.59+	6.35+	11.00+
From incubation medium	0.120	0.34	1.46
Sediment at 1,000g	4.06	9.20	10.65
Sediment between 1,000g and 10,000g	7.74	17.1	24.7
Supernatant at 10,000g	4.65	8.28	18.1

+ These values represent incorporations of 6.67%, 14.3% and 18.2% of ^{14}C-leucine added to incubations.

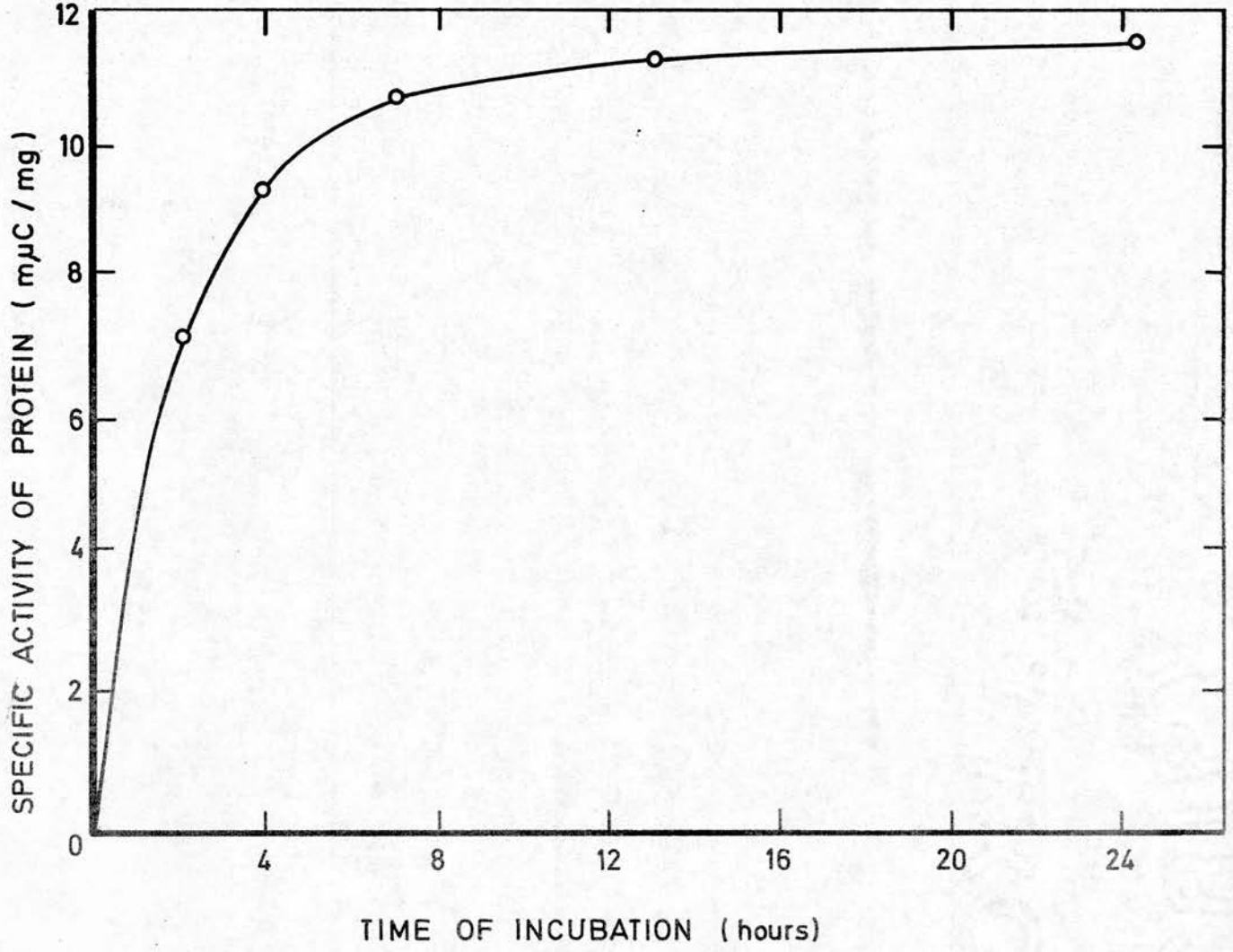


FIGURE 6 TIME DEPENDENT INCORPORATION OF ^{14}C-LEUCINE INTO SHEEP THYROID SLICES.

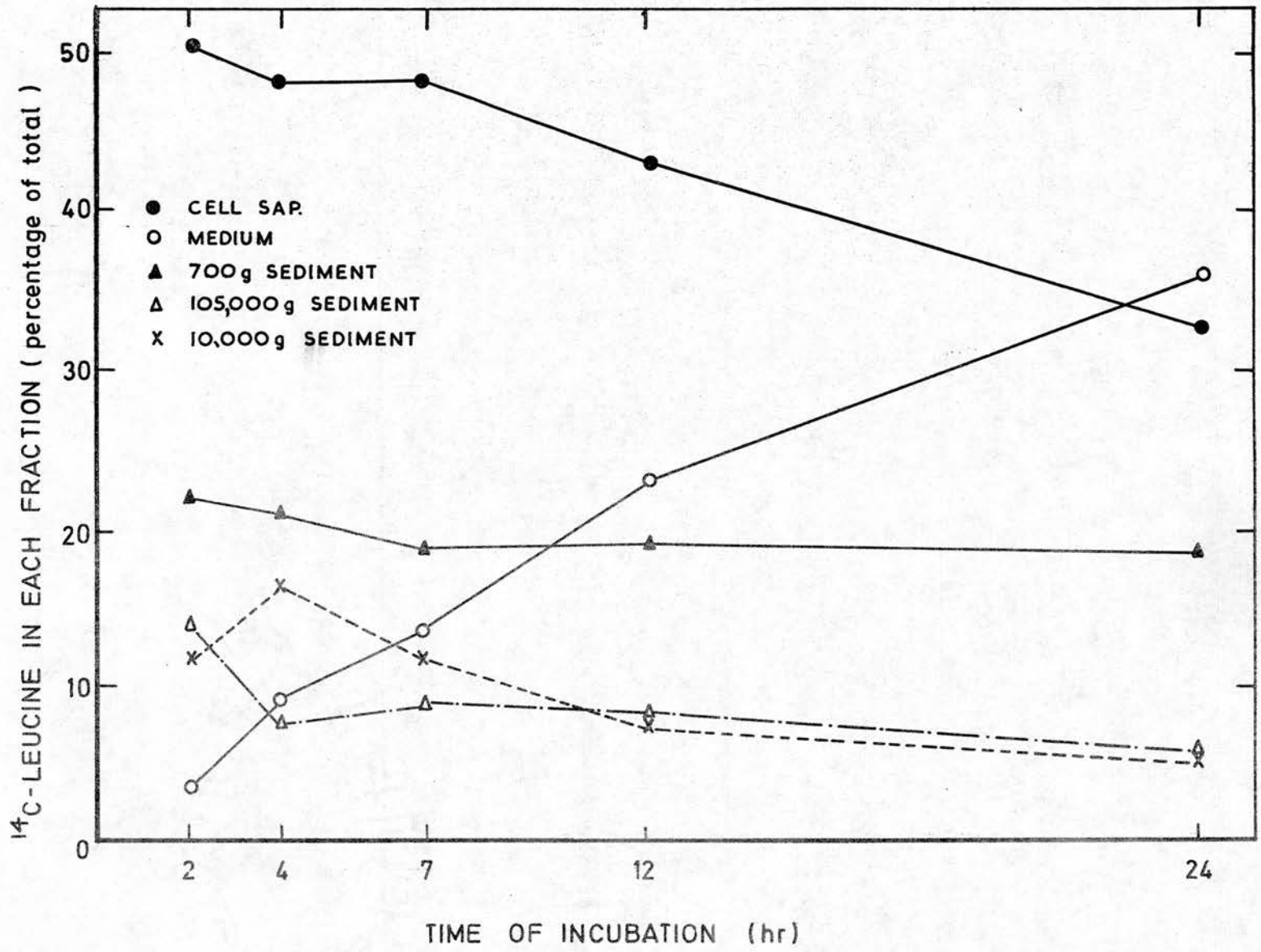


FIGURE 7 TIME DEPENDENT CHANGES IN THE DISTRIBUTION OF ^{14}C-LEUCINE INCORPORATED INTO FRACTIONS OF SHEEP THYROID SLICES.

An initial experiment demonstrated that the incorporation of ^{14}C-leucine into the protein of all thyroid fractions increased with time.

Portions (100 mg.) of sheep thyroid slices were incubated with 1.67 μC of ^{14}C-leucine in 1.1 ml. of KRB for 1, 2, and 3 hr. The specific activities, mμC/mg., of the protein in the incubation medium, and of the fractions corresponding to cell debris, mitochondria, and cell sap plus microsomes were found (Table 18), (following p. 53).

The initial experiment was extended to find (a) how long the apparently linear uptake of ^{14}C-leucine continued, and, (b) if the longer incubation resulted in different levels of incorporation into different fractions.

As previously, 100 mg. portions of sliced thyroid were incubated in 1.1 ml. of KRB with 1.67 μC of ^{14}C-leucine. The incubation medium, the particulate fractions sedimenting at 700 *g*, 10,000 *g*, 125,000 *g*, and the post-microsomal supernatant were assayed for radioactivity. Both the fractions sedimenting at 700 *g* and 10,000 *g* were washed by resuspension and recentrifugation.

The rate of incorporation of ^{14}C-leucine into the total protein from each thyroid decreased with time becoming zero after approximately 13 hr. (Fig. 6).

However, the distribution of label among the fractions did not remain constant. The percentage of incorporated activity in the cell sap protein, constant over the preceding 5 hr., fell after 7 hr. This was accompanied by an increase in the radioactivity of the protein in the incubation medium.

This process (shown in Fig. 7) apparently represents the incorporation of ^{14}C-leucine into protein on the particulate fraction of the cells followed by solubilization of the protein and its transport

Table 19. Incorporation of ^{14}C-leucine by sheep thyroid slices

Sliced sheep thyroid (100 mg.) incubated in 1.10 ml. of KRB with 1.67 μC of ^{14}C-leucine.

Protein fraction	Specific activities of protein isolated (mμC/mg. protein)				
Time of incubation (hr.) -	2	4	7	13	24
700g sediment	24.5	49.0	13.3	16.1	16.6
10,000g sediment	7.10	13.9	19.5	6.84	6.78
125,000g sediment	8.00	4.94	7.74	8.08	6.22
125,000g supernatant	8.60	11.3	12.9	16.7	13.6
Incubation medium	0.92	2.68	5.12	7.22	10.9
Whole thyroid+	7.22	9.33	10.6	11.1	11.5

+ These values represent incorporations of 14.2%, 20.3%, 25.6%, 23.5% and 26.4% of the ^{14}C-leucine added to the incubations.

Table 20. Time-dependent efflux of labelled protein into incubation medium

Time of incubation (hr.)	2	4	7	13	24
^{14}C-leucine in protein (mμC)	12.1	44.2	81.5	128	239
Weight of protein (mg.)	13.1	16.7	15.8	17.7	21.7
Specific activity (mμC/mg.)	0.92	2.65	5.16	7.26	11.02

to the follicles, many of which, in this preparation, will be open to the medium. Table 19 shows the specific activities of the fractions isolated.

Closer examination of the specific activity of the protein in the incubation medium (Table 20) reveals that the rise in weight, from 2 hr. to 24 hr., 8.6 mg., was accompanied by a rise in incorporation of 227 mμC. The specific activity of the protein entering the incubation medium after 2 hr. was 26.4 mμC/mg.

From this it appears possible that preincubation of the slices with or without ^{14}C-leucine in the medium will result in the early loss of pre-synthesized non-labelled protein from the follicles and cell cytoplasm. Labelled thyroglobulin has been found to appear in the lumen of the follicles 4 hr. after the addition of ^{14}C-amino acid, (Salvatore et al., 1965).

II.4.2. Effect of preincubation of slices with ^{14}C-leucine on incorporation: To test the hypothesis that the protein which passed into the medium in the early part of incubation would consist largely of preformed unlabelled material, four identical incubations were set up containing approximately 100 mg. tissue and 1.67 μC in 1.1 ml. KRB. The preliminary incubations were continued for 4 hr. 35 min. After cooling, the slices were removed, gently blotted and added to four identical incubations, which were 'gassed' and then continued for 4 hr., 9 hr., 19 hr., and 30 hr. respectively. Fractions isolated from the slices were assayed for radioactivity. The incubation media contained an increasingly large quantity of sediment which was removed by centrifugation and treated separately.

Table 21 shows increased activity in most fractions, but an especially large increase in the specific activity of the soluble protein in the incubation medium. In this experiment, however, the

Table 21. Effect of pre-incubation on time-dependent uptake of ^{14}C-leucine by sheep thyroid slices

Sliced sheep thyroid (100mg#.) incubated in 1.10 ml. of KRB with 1.67 μC of ^{14}C-leucine.

Protein fraction	Specific activity of isolated protein (mμC/mg.)				
Time of incubation (hr.) -	4.4	8.4	13.4	23.4	34.5
Pre-incubation medium	3.03	-	-	-	-
Sediment in incubation medium	-	9.3	17.2	25.0	38.2
Soluble protein in incubation medium	-	10.8	18.2	21.5	80.0 (24.4)+
700g sediment	-	19.0	25.0	20.6	78.8
10,000g sediment	-	41.0	24.1	25.6	99.1
105,000g sediment	-	40.4	47.6	77.8	94.0
105,000g supernatant	-	51.2	43.2	43.2	36.0
Total protein	-	21.7	20.1	21.6	29.2

+ Purification of a large or small sample of protein led to different specific activities. This effect is examined later, p. 57.

Table 23. Incorporation of ^{14}C-tyrosine by sheep thyroid slices

See Section II.4.4 for details of incubation.

Protein fractions	Specific activity of protein (mμC/mg.)
Incubation medium	5.6
Incubation medium soluble in 42% A.S.	9.4
Incubation medium insoluble in 42% A.S.	16.6
Post-microsomal supernatant	19.4
Post-microsomal supernatant soluble in 42% A.S.	16.8

Table 22. Effect of pre-incubation on uptake of ^{14}C-leucine by sheep thyroid slices

Details of incubation conditions in Section II.4.3

Source of protein fractions	Specific activity of protein (mμC/mg.)		
	(a)	(b)	(c)
Pre-incubation media	-	1.9	-
	-	3.0	-
Incubation media	11.2	18.6	9.0
	8.6	26.5	11.0
10,000g sediment	22.1	26.9	39.6
	19.5	24.5	43.5
125,000g sediment	5.2	12.6	7.8
	6.8	9.8	8.3
125,000g supernatant	7.1	14.0	10.8
	9.4	15.4	12.4
Total protein	8.14	16.8	12.6
	8.26	19.1	13.8

Table 24. Incorporation of ^{14}C-arginine by sheep thyroid slices

Details of incubation conditions in Section II.4.6

Source of protein fractions	Protein wt. (mg.)	Specific activity of protein (mμC/mg.)	Activity/ fraction (mμC)
Incubation medium	26.7	37.7	1,000
700g sediment	13.2	87.1	1,150
10,000g sediment	7.5	88.0	660
105,000g sediment	10.8	31.5	340
105,000g supernatant	39.9	51.9	2,070

specific activity of this protein did not rise above that of the cell sap protein. From Table 21 much of the activity in the cell sap protein appears to have transferred to the insoluble protein in the incubation medium. This procedure of preincubation has more than doubled the specific activity of the protein isolated.

Samples of cell sap protein from the slices incubated for 4 hr. and 19 hr. were eluted from Sephadex G-200 (see Section III.2.2. for details) and shown to contain very largely pure thyroglobulin.

II.4.3. Preincubation with and without ^{14}C-leucine: To continue the investigation of the preincubation procedure three incubations were set up in duplicate. The first of these, (a), was preincubated in 1.10 ml. KRB, and the others, (b) and (c), in 1.10 ml. KRB with 1.67μC ^{14}C-leucine for the same time. After 4 hr. 18 min. incubation the slices from (a) and (b) were transferred to 1.10 ml. KRB containing 1.67μC ^{14}C-leucine, regassed and the incubation continued. Incubation (c) was merely regassed. All incubations were continued for a further 12 hr.

The specific activity of the protein fractions was highest where preincubation had been done in the presence of ^{14}C-leucine. This was shown by the total uptake per incubation, and also by the specific activity of the total protein (Table 22).

II.4.4. Preparation of thyroglobulin labelled with ^{14}C-tyrosine: Using the above preincubation conditions larger amounts of sliced thyroid were incubated with ^{14}C-tyrosine. The greater yield of soluble protein meant that ammonium sulphate fractionation could be more easily achieved.

Sheep thyroid slices (638 mg.) were added to 6.60 ml. of KRB containing 10μC ^{14}C-tyrosine. The preincubation under an atmosphere of O_2:CO_2 (95%:5%) was continued for 4 hr. At this time

the slices were chilled and transferred to an identical incubation for a further 9 hr.

The preincubation, incubation and cell sap proteins were partially fractionated with ammonium sulphate. Table 23 lists the specific activities of the isolated protein fractions.

The weights of protein in the preincubation and incubation media, based on the weight of protein isolated from samples of these, were 88 and 68 mg. respectively. The total weights of protein from these fractions, soluble and insoluble at 42% saturated ammonium sulphate, were 27.7 and 32.6 mg. This loss of thyroglobulin was investigated and the results, and conclusions, shown in Table 25.

II.4.5. Purification of thyroid protein by dialysis and freeze-drying: All the protein fractions from the incubation medium and the cell sap were pooled and dialysed against 0.1N NH_4OH to remove the ammonium sulphate. After dialysis, the protein was freeze-dried and a small portion was weighed and counted. The specific activity was 65 mμC/mg. - the highest value yet found. This value after dialysis, is in contrast to the specific activities listed in Table 23 which range from 5.6 to 19.4 mμC/mg. of the same protein. This effect was thought to be produced by the removal of salt-like material during dialysis thus increasing the specific activity of the remaining protein.

II.4.6. Incorporation of ^{14}C-arginine into thyroid protein: Under the conditions used previously 621 mg. of sliced sheep thyroid was preincubated with 10 μC of ^{14}C-arginine in 6.6 ml. KRB. The second incubation, for 9½ hr., also contained 10 μc ^{14}C-arginine.

Under identical conditions protein fractions were labelled to a greater degree with ^{14}C-arginine than previously with ^{14}C-tyrosine

Table 25. Effect of method of purification on yield and activity of protein labelled with ^{14}C-leucine by pre-incubation

For conditions of incubation and purification see Section II.4.7

Source of protein fractions	Apparent wts. of protein before and after dialysis (mg.)		Apparent specific activities before and after dialysis (mμC/mg.)	
i) Pre-incubation medium	140	57.3	2.00	11.0
ii) Incubation medium	74	26.2	32.3	136
iii) 10,000g sediment	36.5	-	90.5	-
iv) 10,000g supernatant	181	116	37.6	118
v) Mixed samples of ii) & iv), i.e. protein already exhaustively purified	20.8	11.2	36.3	123

Table 27. Effect of increased concentration of ^{14}C-amino acids on the labelling of thyroglobulin

Incubations comprised (A) 1.5 g. of tissue in 15 ml. of KRB containing 25 μC of ^{14}C-amino acid, and (B) 150 mg. of tissue in 1.50 ml. of KRB containing 25 μC of ^{14}C-amino acid.

Source of label	Specific activity of thyroglobulin (mμC/mg.)	
	A	B
Arginine	37.6	404
Leucine	55.1	505
Tyrosine	46.1	231

(Table 23). This is probably due to the higher concentration of arginine in thyroglobulin. Of the 5.22 μC incorporated, 3.07 μC (59%) was present in the soluble protein with a specific activity of 49.6 mμC/mg.

A portion of the incubation medium, which had not been precipitated by trichloracetic acid was dialysed and freeze-dried. This had a specific activity of 273 mμC/mg. Chromatography of this sample revealed that it contained no ^{14}C-arginine removable by butanol:acetic acid.

II.4.7. Preparative incorporation of ^{14}C-leucine into sliced sheep thyroid: Approximately 1.6 g. of sliced sheep thyroid was pre-incubated in 16.4 ml. of KRB containing 23.3 μC of ^{14}C-leucine for 4 hr. The second incubation in 16.6 ml. KRB containing 26.7 μC of ^{14}C-leucine continued for 11 hr. Samples of the protein fractions (Table 25) were purified and assayed for radioactivity. The bulk of the fractions were dialysed separately, freeze-dried and counted without further purification. The samples, which had been rigorously purified and assayed, were pooled, freeze-dried, weighed and counted.

Table 25 contains the weights and specific activities of the total amounts of protein in each fraction as estimated on the basis of samples from these fractions. In each case the estimate after dialysis and freeze-drying is lower and the specific activity higher. Most striking is the loss of weight of the already thoroughly purified samples accompanied by a rise in specific activity to the level of the freeze-dried samples.

This can be explained by contamination of the purified protein with a considerable quantity of material, such as salt, which not only spuriously increases the protein weight but also causes a

Table 26. Increased ^{14}C-activity of thyroglobulin produced by pre-incubation of thyroid slices

All samples of thyroglobulin were dialysed and freeze-dried before weighing and counting. Samples 4, 5 and 6 were counted after the ^{131}I-activity was negligible. Incubation conditions were :-

1) 638 mg. of tissue in 6.60 ml. of KRB containing 10 µC of ^{14}C-tyrosine for 4 hr. (pre-incubation) and an identical incubation for a further 9 hr.

2) 1.60 g. tissue in 16.4 ml. of KRB containing 23.3 µC of ^{14}C-leucine for 4 hr. (pre-incubation) and an identical incubation with 26.7 µC for a further 11 hr.

3) 621 mg. of tissue in 6.60 ml. of KRB containing 10 µC of ^{14}C-arginine for 4 hr. followed by an identical incubation for a further 9 hr.

4) 150 mg. of tissue in 1.20 ml. of KRB containing 47 µC of ^{14}C-tyrosine and 25 µC of $^{131}I^-$ for 8 hr.

5) 177 mg. of tissue in 1.15 ml. of KRB containing 26.5 µC of ^{14}C-arginine, 33.5 µC ^{14}C-leucine, 24.5 µC ^{14}C-tyrosine and 25 µC of $^{131}I^-$ for 8 hr.

6) 150 mg. tissue in 1.50 ml. of KRB containing 100 µC of ^{14}C-algal protein hydrolysate and 25 µC of ^{131}I for 8 hr.

Source of label	Specific activity of thyroglobulin (mµC/mg.)
1. Tyrosine	78
2. Leucine	142
3. Arginine	273
4. Tyrosine	300
5. Arginine, leucine and tyrosine	780
6. Algal protein hydrolysate	1,140

large degree of self-absorption. The increased activity and decreased weight of the already purified sample can only be explained in this way. The salt presumably fails to dissolve in the organic solvent mixture used to wash the protein after its precipitation from 2N NaOH by 6N HCl.

Further labelling using different sources of carbon-14 was carried out with the revised conditions for incorporation. A ^{14}C-algal protein hydrolysate was used with the intention of labelling most of the amino acids in thyroglobulin. Another incubation was used to label thyroglobulin simultaneously with ^{14}C-arginine, ^{14}C-tyrosine and ^{14}C-leucine. By using increased levels of label the specific activity of iodotyrosyl peptides was considerably raised.

Duplicate samples of the dialysed, freeze-dried thyroglobulin, labelled under the preincubation conditions, were weighed on a torsion balance and counted (Table 26). The level of activity made both the detection of peptides and the determination of their constituent amino acids feasible.

II.4.8. Raised ^{14}C-amino acid concentration in incubation medium leading to raised specific activity of isolated protein: The incorporation of amino acids into protein by the thyroid-trachea preparation had been approximately proportional to the concentration of the amino acid in the medium. By decreasing the quantity of sliced thyroid and medium, while maintaining the same quantity of amino acid, incorporation was enhanced.

Two series of incubations were set up. In (a) the above conditions were used, i.e. 1.5 g. tissue, 15 ml. medium and 25 μC ^{14}C-amino acid. In (b) 150 mg. of slices, 1.15 ml. KRB and 25 μC ^{14}C-amino acids were made up. Incubation was continued for 9½ hr.

with one 'gassing' after 4 hr.

The specific activities of the soluble proteins are listed in Table 27.

Approximately 10% by weight of the slices was soluble protein. The right-hand column indicates a percentage incorporation of between 15% and 32% of total amino acid into the soluble fraction with the higher level of ^{14}C-amino acids.

II.5. In vitro iodination of sliced sheep thyroids

Later stages in the research required thyroglobulin labelled with $^{131}I^-$ so that the peptides concerned in thyroxine synthesis might be identified. This labelling was done under conditions identical with the incorporation of ^{14}C-amino acids into thyroglobulin. Advantage was taken of the ease with which thyroid slices will concentrate iodide from a suitable medium.

1.03 g. of sliced sheep thyroid was added to 11.13 ml. KRB containing 200 μC of $Na^{131}I^-$. After 3 hr. the flask was regassed and the incubation was continued for 8 hr.

The slices were homogenized in the incubation medium. The supernatant from two sedimentations at 15,000 g for 20 min. was dialysed against three changes of 2 l. of 0.1N NH_4OH. During dialysis the diffusate was checked each time to estimate the loss of $^{131}I^-$ from the dialysis sac. The protein was finally freeze-dried.

All further in vitro iodination of sheep slices was carried out as above. The relative concentration of tissue to medium was constant although the quantity of $^{131}I^-$ added varied, as did the total size of the incubation. Incubations which were continued for 24 hr. had two drops of toluene added as a bacteriostat.

II.6. Incorporation of $^{131}I^-$ and ^{14}C-amino acids into sliced thyroids

In the production of doubly-labelled thyroglobulin the amino acid(s) and iodide were added together to the KRB before incubation. The protein was isolated, dialysed and freeze-dried as usual.

Chapter III

PURIFICATION AND ANALYSIS OF THYROGLOBULIN PREPARATIONS

Later investigations (Chapters IV and V) are concerned with the specific parts of the thyroglobulin molecule where iodination of tyrosyl residues occurs. These specific regions, namely the iodotyrosyl residues and the surrounding peptide chains, were to be isolated by peptide-mapping after α-chymotryptic hydrolysis of thyroglobulin. For ease of detection these peptides must have a sufficiently high specific radioactivity.

Thyroglobulin labelled with ^{14}C-amino acids, isolated as described in Section II.3.4. from surviving thyroid tissue, contains all the soluble thyroidal proteins. Peptides released from non-thyroglobulin protein during peptide mapping might be sufficiently radioactive to be detected by autoradiography and to interfere with the visualisation of the thyroglobulin peptides. The extent of label in contaminating peptides would depend on (a) the rate of synthesis of the particular protein, i.e. the rate of incorporation of a labelled amino acid, (b) the quantity of this protein present, and (c) the possibility that several proteins might yield common peptides.

It was necessary to find the purity of thyroglobulin, as isolated, and to increase this purity to an acceptable level. Previously thyroglobulin has been most often purified by ammonium sulphate fractionation (Derrien, Michel and Roche, 1948, and Ui and Tarutani, 1961). Another preparative method was chromatography on DEAE-cellulose (Lissitsky, 1966). Since thyroglobulin salts-

out between sharply-defined limits, fractionation with ammonium sulphate was the first technique applied. Adsorption chromatography was not used because, unlike salting-out which depends only on protein solubility, on DEAE-cellulose 'pure' thyroglobulin separates into fractions differing only in the relative content of iodine. Purification of thyroglobulin by gel filtration on Sephadex G-200 which depends solely on molecular size, was expected to be an effective technique - thyroglobulin being much larger than the majority of cellular proteins. The purity of different thyroglobulin preparations was examined by gel filtration, electrophoresis on starch or acrylamide gels or cellulose acetate paper, sucrose gradient and moving boundary ultracentrifugation, and amino acid analysis.

Fractionation of soluble thyroid proteins with ammonium sulphate was achieved readily with decigram quantities of starting material. Over 80% of protein extracted from sliced thyroid precipitated between the limits of 37% and 43% saturated ammonium sulphate indicating that this preparation was largely thyroglobulin.

However, this technique, when applied to the small quantities of protein from rat thyroid, failed to produce fractionations that were consistent, leading to the loss of labelled thyroglobulin.

Gel filtration on Sephadex G-200 was used, initially to find the quantity of thyroglobulin which did not appear in the 'thyroglobulin' fraction of the ammonium sulphate preparation. As much as 60% of thyroglobulin was lost in this way.

Gel filtration proved to be an easy method, not only for analysing protein solutions, but also for preparing sizeable (20 mg.) quantities of protein. Thyroglobulin with its large molecular weight elutes well ahead of the majority of cellular

proteins.

Gel filtration of the soluble protein from *in vitro* incubations of sheep thyroid slices revealed that it contained a very high percentage of thyroglobulin. It was dialysed and freeze-dried. This thyroglobulin preparation was sufficiently pure to be used as the starting material for peptide mapping.

Nevertheless, analyses, by techniques mentioned above, were carried out to check the purity of this and other thyroglobulin preparations.

III.1. Ammonium sulphate fractionation

III.1.1. Method: The protein solutions to be fractionated were prepared by an 0.9% NaCl extraction of sliced thyroid or by removal of sub-cellular particulate material from thyroid homogenates by centrifugation at 125,000 *g* for 40 min.

Fractionation followed the procedure of Derrien, Michel and Roche (1948) using an ammonium sulphate solution saturated at room temperature (18°) and having a pH of 7. Thyroglobulin in the crude extract was precipitated in 45% saturated ammonium sulphate produced by the addition of the required quantity of the saturated salt solution. After standing for 1 hr. the precipitate was centrifuged off at 5,000 *g* and resuspended in 45% ammonium sulphate. The solution was diluted with water to 37% saturation, allowed to stand for 1 hr. or longer and centrifuged as before. The supernatant concentration was increased to 41% and the precipitated protein removed by centrifugation. Shulman and Armenia (1963) found that this type of preparation contained 93% to 97% thyroglobulin.

In some cases the limits of fractionation were widened to between 35% and 45% or 37% and 42% saturated ammonium sulphate,

Table 28. Ammonium sulphate fractionation of small quantities of (a) pig, and (b) sheep thyroid protein

	Protein fractions	Protein weight (mg.)	
(a)	Soluble in 45% A.S.*	6.1	
	Insoluble in 35% A.S.	7.8	
	Thyroglobulin	19.5	(58%)
(b)	Soluble in 42% A.S.	18.3	
		17.2	
	Insoluble in 42% A.S.	7.6	
		13.0	

Table 29. Ammonium sulphate fractionation of the protein from a saline extract of rat thyroids

Limits of solubility of protein fractions	Protein weight (mg.)
42% A.S. - 10% TCA+	11.3
0 - 37% A.S.	9.1
41 - 42% A.S.	5.3
37 - 41% A.S.	5.3

Table 30. Ammonium sulphate fractionation of the cell sap protein from rat thyroids

Protein weight per fraction (mg.)			
Limits of solubility			
42% A.S. - 10% TCA	0 - 37% A.S.	Thyroglobulin[x]	(37 - 42% A.S.)
3.7	0.8	0.92	(20%)
1.1	0.7	0.88	(19%)
3.2	0.5	1.12	(23%)
3.3	0.5	0.56	(13%)
2.5	0.1	0.11	(4%)
4.0	-	0.60	(13%)
45% A.S. - 10% TCA	0 - 35% A.S.[x]	Thyroglobulin	
5.6	0.18	3.4	(43%)
6.8	0.13	2.6	(29%)

* $(NH_4)_2SO_4$

+ Trichloracetic acid

x Determined by extinction at 280 mμ.

because the small volume of solution made accurate concentration and dilution difficult. The fractions were occasionally dialysed before undergoing purification for counting and always before freeze-drying.

III.1.2. Results:

III.1.2.1. Fractionation of sheep thyroglobulin: A saline extract prepared from sliced new lamb thyroids was fractionated between the limits of 37% and 42% saturation with ammonium sulphate and the protein in each fraction was estimated spectrophotometrically at 280 mμ using an extinction coefficient ($E^{1\%}_{1cm}$) of 10.0 (Salvatore, Salvatore, Cahnman and Robbins, 1964). The quantities of protein separated were 8.91 mg. soluble at 42%, 5.99 mg. insoluble in 37%, and 66.76 mg. of thyroglobulin. This saline extract contained over 80% of thyroglobulin, in agreement with reported values.

Fractionation of smaller quantities of protein proved to be less accurate. Protein from media in which pig or sheep thyroid slices had been incubated was fractionated, purified and weighed (Table 28). The yield of thyroglobulin was less than the expected 80% of the total protein. This point is dealt with in the discussion in Dixon and Webb (1961).

III.1.2.2. Fractionation of rat thyroglobulin: The technique of saline extraction of sliced tissue followed by ammonium sulphate fractionation was applied to rat thyroids which had been incubated *in vitro* with ^{14}C-leucine as part of the thyroid-trachea preparation. The distribution of protein in a saline extract of four thyroids is shown in Table 29. Fractionation of the cell sap from such rat thyroids also showed an unexpectedly low yield of thyroglobulin (Table 30).

Much of the protein had not precipitated at the upper level of ammonium sulphate concentration. To discover whether any of this

protein was thyroglobulin, isolated fractions were chromatographed on Sephadex G-200 (for conditions, see Section III.2.2.).

Six rat thyroids (144 mg.) were incubated in KRPB-III with 0.25 μC of ^{14}C-leucine. After 3 hr. the glands were removed from the tracheae, washed and homogenized in 0.9% NaCl. The homogenate was centrifuged twice at 10,000 g for 20 min. and the supernatant fractionated into protein soluble and insoluble at 45% ammonium sulphate. The insoluble protein was dissolved in Tris-KCl and run on a G-200 column of 234 ml. bed volume. The soluble fraction was run on a column of volume 225 ml. Fractions from the column (2 ml.) were assayed for protein by the absorption at 280 mμ. Both elution patterns showed a large main peak eluting at the position of thyroglobulin.

Of the thyroglobulin eluted there was more (4.77 mg.) in the protein soluble in 45% ammonium sulphate, than in the protein insoluble at this concentration (3.56 mg.). A repeat of this experiment using smaller quantities of protein yielded 0.28 mg. insoluble and 0.27 mg. soluble in 45% ammonium sulphate.

Although a measure of purification was achieved, this was not attained without considerable loss of thyroglobulin. As the amount of labelled protein was very limited, this method of purification was discarded.

III.2. Fractionation and analysis of thyroglobulin preparations on Sephadex G-200

III.2.1. Preparation of columns: Sephadex columns were prepared by a method based on that of Andrews (1964).

Each column was packed in a 70 cm. x 2.82 cm. glass tube. The outlet from the column was constricted to decrease the dead space.

The outlet was attached to polypropylene tubing (Sterivac, Allen and Hanbury, 1.5 mm. bore) and an easily controlled flow was achieved by partially clamping this. The Sephadex column was supported on a perforated porcelain disc covered with a pad of glass wool on which a layer of coarse sand was formed by adding the sand through a column of water. This was followed by a layer of fine sand to give a total depth of sand of 3-4 mm. In the absence of sand the Sephadex filtered through the glass wool. The sand was cleaned by soaking in chromic acid (10% (w:v) solution of potassium dichromate in concentrated sulphuric acid) followed by NaOH (10%) and several washes in tap water and finally in distilled water. After drying, it was sieved into coarse and fine grades.

G-200 Sephadex (7 g.) swollen in water over 2-3 dy. had been rinsed in large volumes of water to remove as many of the fines as possible. The gel was added as a slurry to the water-filled column. About 1-2 in. were allowed to settle before the clamp was opened very slightly. When most of the gel had settled some of the supernatant in the column was removed along with any fines in it and more slurry was added. This process was continued until all the gel had been added to the column. The columns were packed under a head of about 20 cm. It was essential that the columns were vertical during packing or the surface of the gel became tilted giving a larger spread of peaks during elution. Addition of dense slurry to the column set up currents which occasionally led to the same difficulty. This could be avoided by slowly stirring the supernatant in the column.

Finally a circular piece of Whatman No. 1 filter paper of the same diameter as the column was placed on top of the supernatant and allowed to sink down until it rested on top of the gel. A slow

flow rate ensured that the paper settled firmly and horizontally on to the gel. The paper kept the gel undisturbed during the addition of protein solution of eluant.

The top of the column was sealed with a bung through which polypropylene tubing brought the buffer. The tube dipped under the surface of the supernatant and, with the column outlet clamped, any pressure put on the gel by squeezing in the bung was released by the supernatant running back up the delivery tube. The closed system ensured that the rate of inflow and outflow from the column were identical and could be controlled at one point.

Each new column was washed with water for at least 24 hr. under the working pressure head of approximately 30 cm. water at a flow rate of 30 ml. per hr., i.e. 6 ml. per sq. cm./hr., although the columns had maximum flow rates of 86 ml./hr. Before use each column was equilibrated with the buffer which was 0.1M KCl in 0.05M Tris-HCl pH 7.4. When not in use the column was filled with buffer containing phenol (1%) to halt bacterial growth.

III.2.2. Sample application, fraction collection, bed volume and exclusion volume: Sample solutions were applied to the column in two ways. If the volume was large or if the sample was dilute the buffer was allowed to run out of the column until the filter paper on top of the column was just exposed. With the outlet clamped the solution was carefully pipetted on to this and then allowed to run on to the column. When all the solution had run on, a little buffer was added to wash in the sample. This process was repeated and the column was set up for running.

If the sample to be eluted was small and with a concentration greater than 5 mg./ml. (Andrews, 1964), it was layered below the buffer on top of the column with a Pasteur pipette.

The fraction eluting when half the solution had entered the column was taken as the first fraction.

The fraction collector used was an LKB Radirac model fitted with a siphon which collected 2.035±0.086 ml. Protein was estimated on a Unicam S.P. 500 by absorption at 280 mμ, usually in 5 mm. cells which held one 2 ml. fraction.

After each column had been packed and equilibrated the bed volume was calculated and the void and exclusion volumes were found. For example, one column had a calculated bed volume of 218 ml., i.e. from height and area of column. To find the void volume, Vo, and to examine the packing of the column a solution of cytochrome-c was added to the column by layering under the buffer. Cytochrome-c penetrates nearly all of the gel so that its elution volume, Ve, corresponds to the void volume of the column, Vo. Initially the visible band of cytochrome-c on the column was level and narrow although it later became more diffuse.

Cytochrome-c was estimated from the extinction at 408 mμ. It eluted with a peak at fraction 109 corresponding to an elution volume, Ve, of 109 x 2.035±.086, that is, 222±9.4 ml. This value agrees with the calculated bed volume. In a later experiment ammonium sulphate, which was estimated by the quantity of material precipitated by 1 ml. of 5% $BaCl_2$ in 10% HCl, eluted with a peak at fraction 112, that is, with an elution volume of 228±9.6 ml.

The exclusion limit of G-200 for globular proteins is 800,000. Thyroglobulin was taken as being excluded, or very nearly so, by the G-200 column. Ammonium sulphate purified hog thyroglobulin (10 mg./ml.) in Tris-KCl was layered below the buffer and eluted with a peak at fraction 36, i.e. 73.3 ml., on the same column.

This column, of calculated bed volume of 218 ml., had estimated

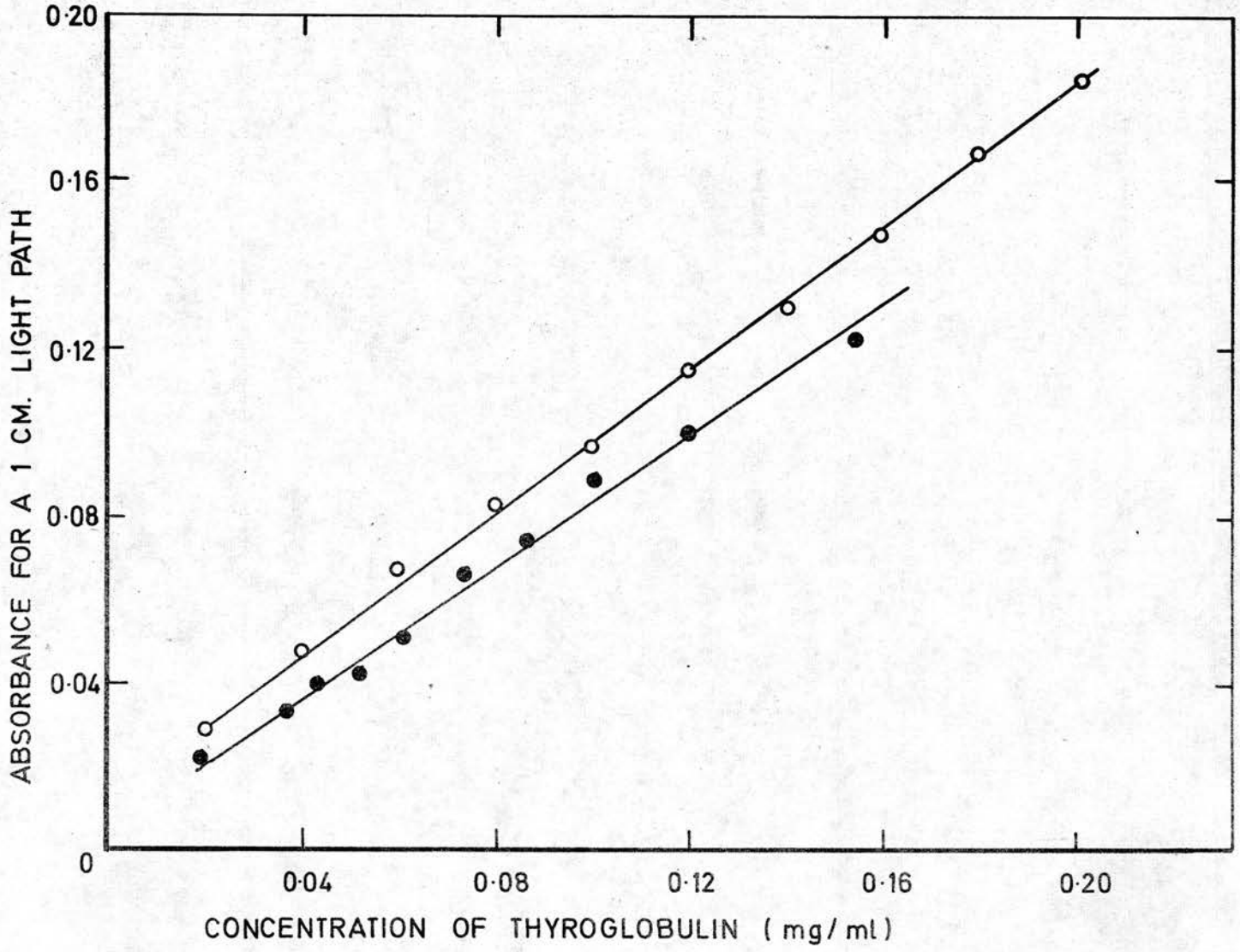


FIGURE 8 ABSORBANCE OF HOG THYROGLOBULIN AT 280 mμ (○—○) AND 260 mμ (●—●)

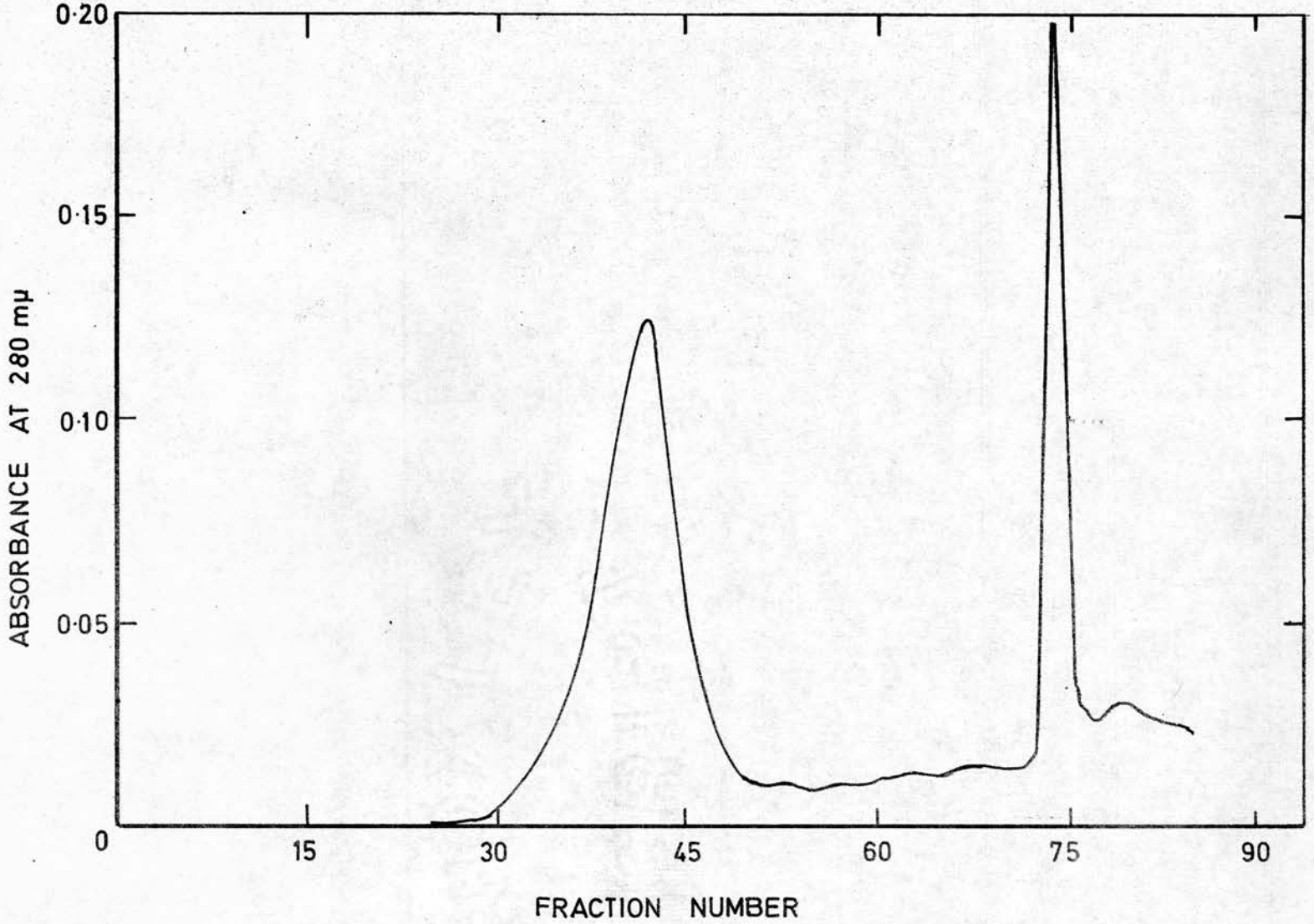


FIGURE 9 ELUTION OF SHEEP THYROID SOLUBLE PROTEINS FROM SEPHADEX G—200.

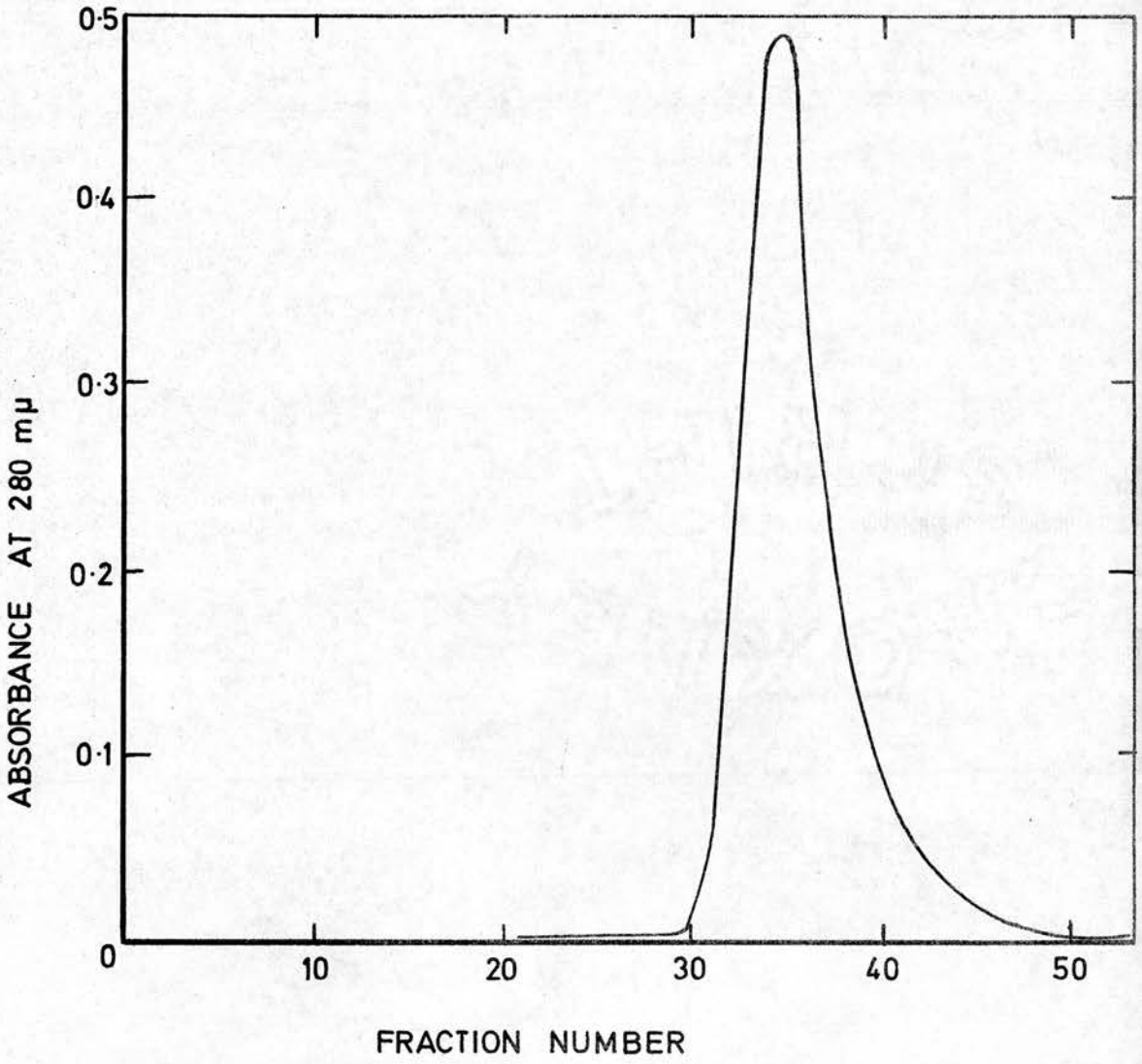


FIGURE 10 STANDARD PREPARATION OF SHEEP THYROGLOBULIN ELUTED FROM SEPHADEX G—200.

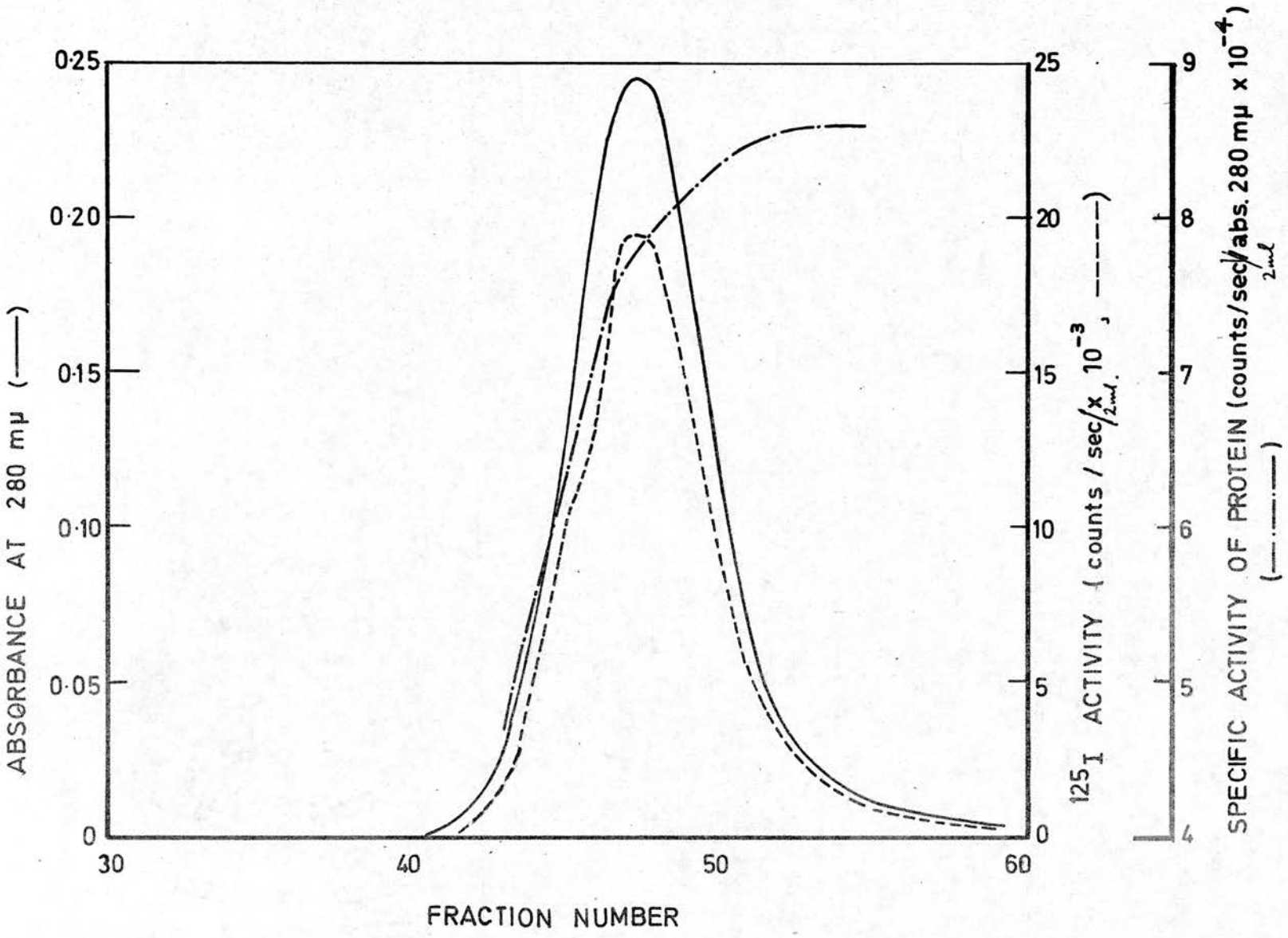


FIGURE II STANDARD PREPARATION OF RAT THYROGLOBULIN, LABELLED WITH IODINE-125 ELUTED FROM SEPHADEX G-200.

bed volumes of 222 ml. (cytochrome-c) and 228 ml. (ammonium sulphate) and an exclusion volume of 73.3 ml. The G-200 dextran has a water regain of 20±2 ml./g., thus the calculated exclusion volume was 218-140 = 78 ml.

III.2.3. Results: Fig. 8 shows the relationship between absorption at 280 mμ and concentration of hog thyroglobulin in 0.05M Tris-HCl, 0.10M KCl buffer pH 7.5. Two separate determinations of $E^{1\%}_{280}$, 1 cm. for thyroglobulin gave 9.7 and 9.1. Salvatore et al. (1964) pointed out that at 280 mμ values ranging from 9.7 to 11.6 (Edelhoch, 1960, Ui and Tarutani, 1961, Shulman, Rose and Witebsky, 1955, and Shulman and Armenia, 1963) have been reported for thyroglobulin of various species and various degrees of purity. In this investigation the specific extinction coefficient of thyroglobulin at 280 mμ has been taken as 10.0.

Fig. 9 shows the high concentration of thyroglobulin in the soluble protein from sheep thyroid slices. The sharp peak at fractions 75 and 76 contained material with a high absorbance at 260 mμ. After dialysis (and freeze-drying) much of the post-thyroglobulin material is lost.

Fig. 10 demonstrates the purity of the standard sheep thyroglobulin preparation from slices homogenized in the incubation medium. From the elution pattern in Fig. 11 the percentage of thyroglobulin present was 91.5%.

Finally the $^{125}I^-$ activity and absorbance at 280 mμ of rat thyroglobulin labelled in vivo with $^{125}I^-$, and prepared and isolated under standard conditions (p.57), is shown (Fig. 11). The ratio of activity to absorption at 280 mμ increases over the peak indicating the protein is not homogeneous with respect to iodine content. This point will be discussed in conjunction with the sucrose gradient work

on p. 79 . The elution pattern also shows that the thyroglobulin is again by far the largest fraction of the soluble protein.

At one stage polyethyleneglycol was used to concentrate the pooled fractions after gel filtration. However, some low molecular weight polymer entered the dialysis sac. When the concentrated dialysis residue was allowed to warm up to room temperature the polymer came out of solution.

III.3. Electrophoresis

III.3.1. Starch gel: As a guide to their purity thyroglobulin preparations were subjected to electrophoresis on supporting media of starch and acrylamide gel and cellulose acetate paper. At the stage of this work when labelled rat thyroglobulin was being prepared it had been considered that these small quantities of material could be purified by preparative electrophoresis on starch gel. When larger quantities of labelled sheep thyroglobulin became available electrophoresis was used solely for analysis.

III.3.1.1. Preparation of gels: Starch, prepared by the method of Smithies (1955) and Poulik and Smithies (1958) was used to prepare gels of 12, 13 and 14% weight/volume. The buffer was 0.2M Tris-HCl of pH 7.5 or 8.6 with ionic strength of 0.049. The gels were set in trays 20 cm. long, 2 cm. broad and 3 mm. deep. Protein was added to the gel as a solution absorbed by pieces of filter paper 17 mm. X 2.4 mm. These were inserted in slits in the gel cut by suitable sized razor blades. The quantity of protein solution absorbed was found by weighing and reweighing the filter paper.

The electrodes attached to a power pack were immersed in two dishes containing the Tris-HCl buffer at 0.3M concentration. These were connected by filter paper wicks to another two dishes containing

the buffer at the same concentration as in the gel. More wicks connected this buffer with the gel. The electrode vessesl contained the products of electrolysis allowing the composition of the buffer in the inner vessesl to remain relatively constant.

During electrophoresis the gel was covered with a plastic sheet to decrease evaporation.

The separated proteins were stained by an 0.5% (w/v) solution of Amido black in methanol:water: acetic acid (5:5:1, v/v). A little of this was added to the surface of the gel which was eased from its Perspex tray, and soaked in more of the dye for 30 sec. Staining was completed by washing the blue gel in the solvent until only the protein remained blue.

III.3.1.2. Results of electrophoresis on starch gel: No satisfactory combination of starch gel concentration and of voltage gradient was found. The bands of thyroglobulin, although apparently uncontaminated by other proteins, were diffuse - an effect probably caused by molecular sieving of the large protein molecule through the concentrated gel or, in addition, by attraction between residual carboxyl groups in the starch and the protein.

III.3.2. Acrylamide gel:

III.3.2.1. Preparation of gels: These were prepared by dissolving 3 g. acrylamide and 15 mg. N,N′-methylene bis acrylamide in 60 ml. of the Tris buffer pH 8.6. To this was added 0.2 ml. of a 10% solution of N,N,N′,N′-tetramethylethylenediamine in ethanol, followed by 0.1 ml. of a 10% aqueous solution of ammonium persulphate. The trays into which the solution was poured were covered with flat perspex slabs and weighted down so that the gels after setting overnight were of uniform thickness. These gels contained only 5% solid material.

Application of protein, electrode assembly and staining were identical with the methods used for the starch gel.

III.3.2.2. Results of electrophoresis on acrylamide gel: A sample of the purified thyroglobulin moved 2 cm. to the anode in a thin band when subjected to 3 volts/cm. for 15 hr. There was one faint band just ahead of the thyroglobulin and another faint band just behind.

A fraction of rat thyroid cell sap precipitating between 35 and 45% saturation with ammonium sulphate was subjected to electrophoresis as above. As well as the major component, the faster and slower moving materials noticed previously were more easily seen. A very small diffuse band of protein 7-10 cm. to the anode may represent contamination with albumin-like material.

The partially purified rat thyroglobulin appears to be only slightly less pure than ammonium sulphate fractionated hog thyroglobulin, which Shulman and Armenia (1963) found to be over 90% pure.

III.3.3. Cellulose acetate paper:

III.3.3.1. Method: Electrophoresis was carried out at pH 8.6 in 0.07M veronal buffer by the method of Smith (1960). The cellulose acetate strips (4.5 cm. x 12 cm.) were floated on the buffer until fully moist, gently blotted and spread between two troughs containing 0.06M veronal buffer (pH 8.6). The current was kept at 0.5 mA./cm. width of paper. The corresponding voltage was between 190-230 volts over 8 cm. length of strip.

Protein solutions (10 μl.) were streaked across the width of the cellulose acetate strip at different distances from the cathodal end whilst the current was flowing to minimise diffusion. The whole apparatus, troughs and strip, was covered by a transparent plastic dome to decrease evaporation from the strip.

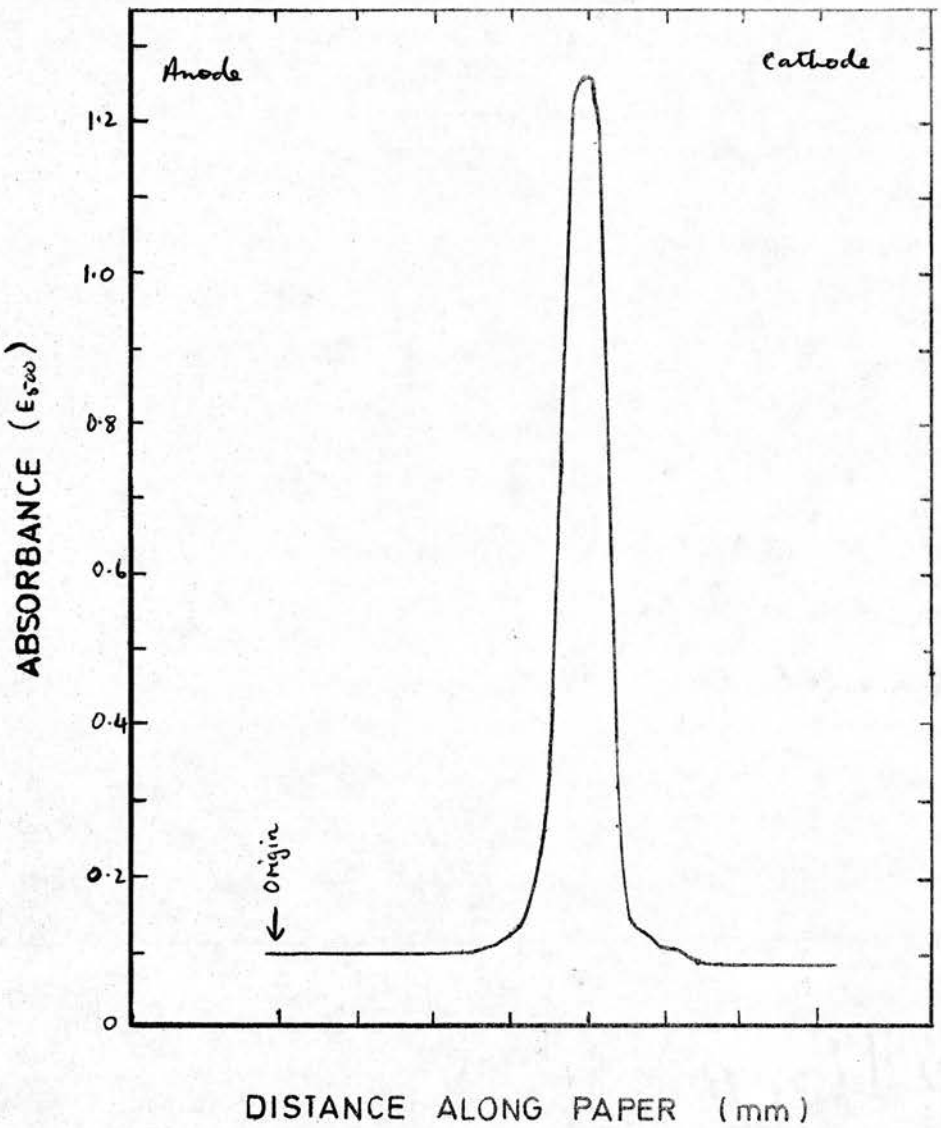


FIGURE 12 ELECTROPHORESIS OF THYROGLOBULIN ON CELLULOSE ACETATE PAPER AT pH 8·6 : SCAN BY TRANSMITTANCE OF THE SOLE PROTEIN BAND STAINED WITH AMIDO-SCHWARZ. Hog thyroglobulin (110 µg) in 10 µl. 0.07M veronal buffer (pH 8·6) was subjected to electrophoresis in the same buffer for 3½ hr. at 26 v/cm.

After electrophoresis the strip was stained for only 5 sec. in Amido black, washed and dried, with pressure, between two sheets of filter paper in a warm atmosphere.

In some cases the dried strips were floated on glycerol until they became almost transparent. The 'cleared' strips were scanned by transmittance in a Chromoscan.

To demonstrate radioactivity on the strips after electrophoresis of labelled protein the dried strips were stapled to X-ray film and exposed for suitable times.

III.3.3.2. Results of electrophoresis on cellulose acetate paper: As indicated in Smith (1960) the initial position of the protein sample on the cellulose acetate strip influenced the mobility of the protein. Hog thyroglobulin (110 μg.) in 10 μl. buffer added to the centre of the strip migrated 0.2 to 0.4 cm. to the anode in 3½ hr. Samples added at positions nearer to the cathode moved under the same conditions over 1 cm. to the anode. This effect is probably produced by a non-linear voltage drop along the paper.

Electrophoresis revealed a single sharp peak, whether application of the solution was made at the centre of the strip or near the cathode (Fig. 12).

To ensure that the procedure was able to separate a mixture of proteins small samples of rat plasma were subjected to electrophoresis for 1½ hr. The optimal time of run was checked by labelling the fast-moving plasma albumin with bromophenol blue so that its migration along the strip could be followed while the electrophoresis was in progress. The major protein components, albumin, α_1-, α_2-, β- and γ-globulin and fibrinogen were clearly separated.

Electrophoresis for 3½ hr. of 150 μg. thyroglobulin labelled with

13,a

13,b

Figure 13. Electrophoresis on cellulose acetate of sheep thyroglobulin labelled with ^{14}C-tyrosine. Sheep thyroglobulin (100/g. in 10/l.) was subjected to electrophoresis in 0.07M veronal buffer (pH 8.6) at 26v/cm. for 12hr. After staining with Amido black a single protein band appeared (13,a). The autoradiogram of the electrophoresis strip also revealed a single band (13,b).

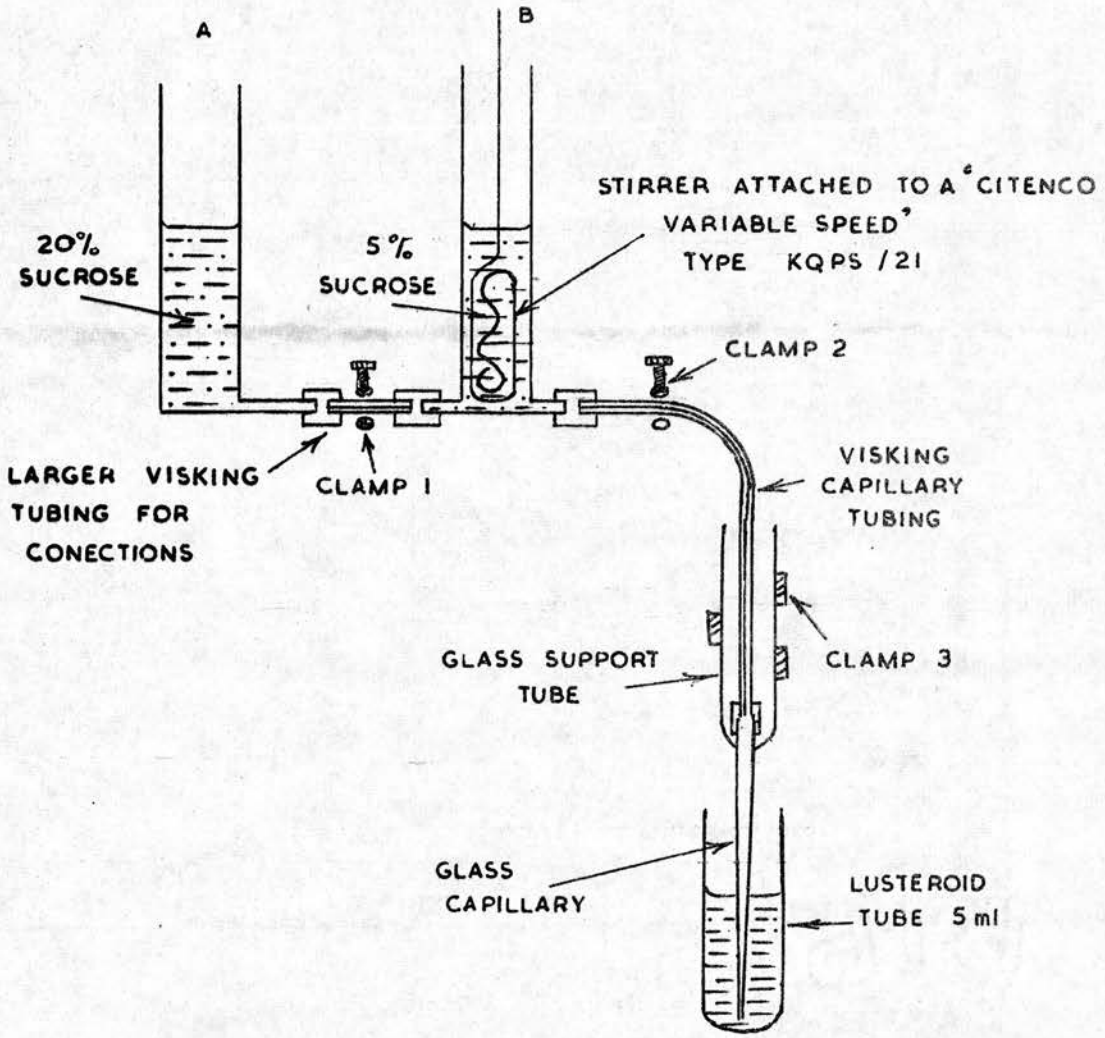


FIGURE 14 APPARATUS FOR THE PRODUCTION OF 5 - 20% SUCROSE GRADIENTS.

^{14}C-tyrosine and purified by the standard method (Section II.4.5.) revealed no contaminating material.

A second electrophoresis using this protein was continued for 12 hr. Staining of the strip showed only one band (Fig. 13,a) and autoradiography for 4 dy. again revealed one band exactly superimposable on the protein band (Fig. 13,b).

Larger samples of protein overloaded the strip giving a less sharp band of thyroglobulin. At concentrations approaching 500 µg. per 10 µl. a faint protein band migrated just ahead of the thyroglobulin.

III.4. Sucrose gradient centrifugation

III.4.1. Preparation of gradients: Sucrose gradient centrifugation was performed essentially as described by Salvatore et al. (1964) and Martin and Ames (1961). Linear sucrose gradients from 5-20% were prepared in 0.05M sodium phosphate buffer, pH 7.4.

The apparatus for making the gradients is shown in Fig. 14. Two flat-bottomed glass tubes of uniform bore were connected by Visking tubing. Tube A contained the 20% sucrose and B the 5% sucrose. Rapid mixing of solutions in B was achieved by an annealed stainless steel wire, twisted into a shape filling the tube, attached to a Citenco Variable Speed Motor Type KQPS/21. The outlet Visking tubing ran through glass tubing so that it could be clamped firmly without decreasing the flow of liquid. The final solution was delivered through a fine glass capillary to the foot of a 5 ml. Lusteroid centrifuge tube. Joins between tubing of different diameters were sealed using cyclohexanol.

Equal volumes of liquid were added to each chamber and clamp 1 (Fig. 14) opened. The two chambers were adjusted until a bubble

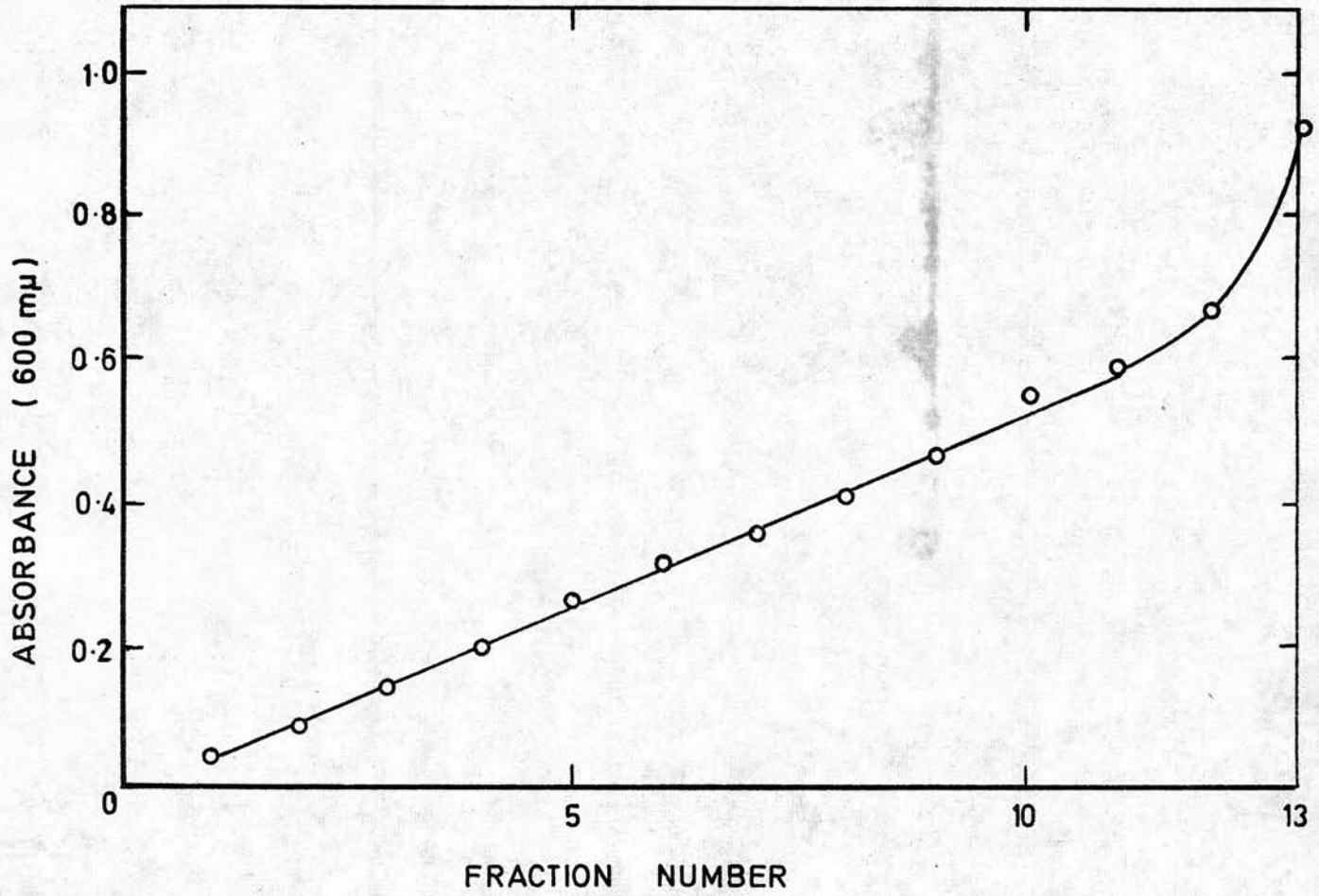


FIGURE 15 PRODUCTION OF A LINEAR 5 — 20% SUCROSE GRADIENT VISUALIZED WITH INK.

trapped in the Visking tubing remained stationary, indicating that the levels in the two chambers were equal.

The apparatus and the sucrose solutions were cooled for several hours at 4°. Sucrose (20% concentration, 2.6 ml.) was added to A and clamp 1 opened until the solution had filled the capillary connecting A to B. Sucrose (5% concentration, 2.7 ml.) was added to B and stirring was started at 200-300 rpm. Clamp 1 was kept closed and clamp 2 was opened until the delivery tube was filled. The heights of the solutions in A and B were now equal.

The delivery tube was set vertically so that the glass capillary was almost touching the foot of the Lusteroid Tube. After opening clamp 1 clamp 2 was gradually opened until a flow rate was established which filled the tube to within 3-4 mm. of the top within 30 min.

Care had to be taken when handling the tube containing the gradient because heat from the fingers or from strong light sources was sufficient to set up rapid convection currents. The tubes containing the gradient, as well as the Spinco rotor SW 39, were allowed to equilibrate at 4° overnight.

To check the linearity of the gradient produced by this apparatus one drop of ink was added to the 20% sucrose. Fractions collected at equal time intervals from the delivery tube were diluted with 3 ml. water and read at 600 mμ (Fig. 15).

III.4.2. Fractionation of the gradients: All fractionation was carried out at 4°. The gradients were removed from the tubes through a fine glass capillary which was inserted vertically, with very little disturbance of the layers, to the foot of the tubes. The Lusteroid tube was held vertically on a small piece of Plasticene. This avoided clamping the flexible tube and ensured full visibility to place the removal capillary centrally at the foot of the tube.

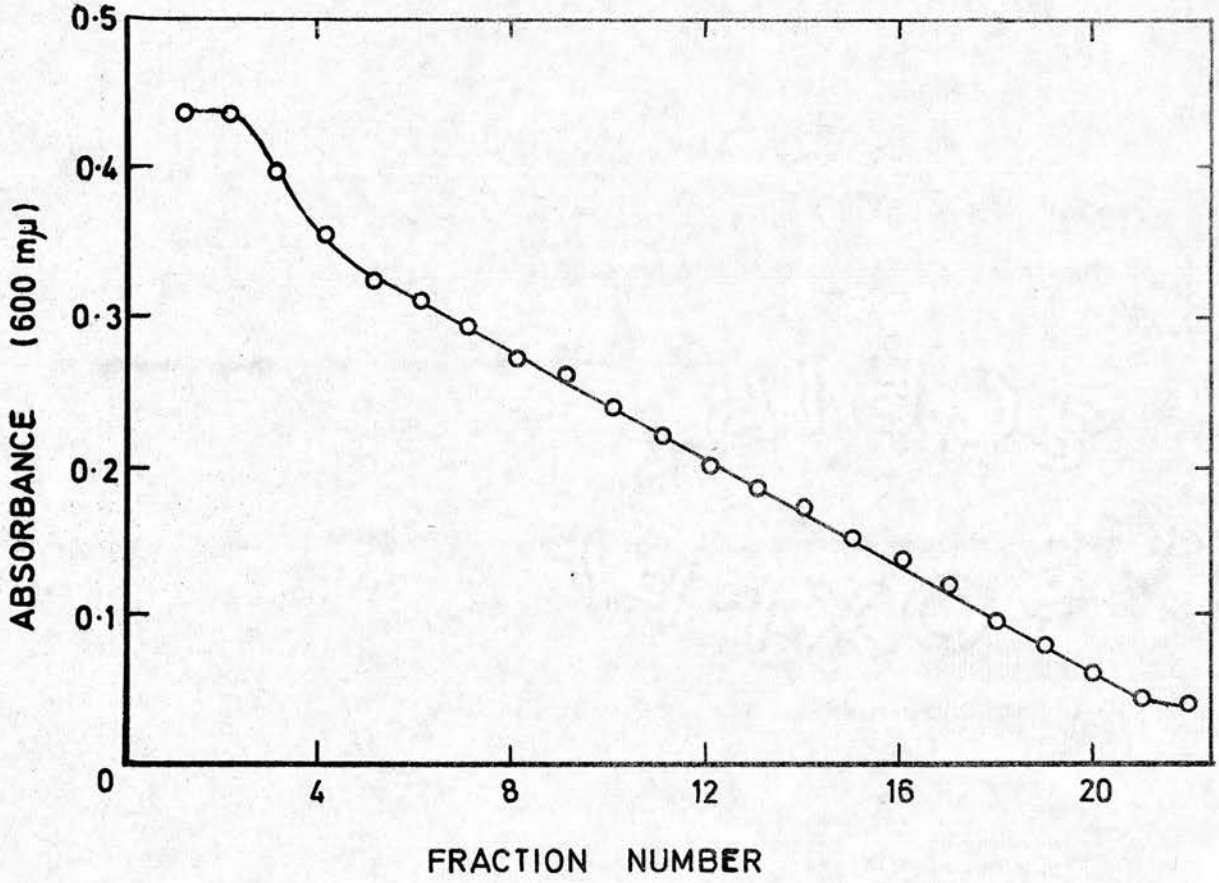


FIGURE 16 FRACTIONATION OF A 5 – 20% SUCROSE GRADIENT VISUALIZED WITH INK.

This capillary was attached to a length of Visking tube of 0.5 mm. bore. A Technicon Autoanalyser proportioning pump removed the liquid at a constant rate of 103 µl./min. This method allowed the continuous removal of liquid with little mixing of fractions.

A gradient was made in a Lusteroid tube in which the 20% sucrose contained a drop of ink. Fractions (206 µl.) were removed from the gradient, diluted with 3 ml. water and read at 600 mµ. A plot of E_{600} against fraction number showed a linear relationship except at the very bottom of the tube (Fig. 16).

Protein solutions were made up in 0.1M KCl - 0.02M Na phosphate and allowed to stand at 4° for several hours. The solutions were then usually centrifuged at 125,000 g for 40 min. to remove any insoluble material.

Approximately 0.75 mg. samples of protein were applied to the top of the gradients in 100 µl. of buffer.

III.4.3. Centrifugation: This was carried out in a Spinco Model ~~E~~L preparative centrifuge rotor SW 39 for 6 hr. or 4 hr. at a nominal 39,000 rpm. The actual rpm was calculated by dividing the difference in odometer readings by the time of run. The actual speed was 40,025 rpm.

III.4.4. Detection of protein and radioactivity in fractions: Total protein was determined at 280 or 210 mµ, or both. At 280 mµ micro cells 2 cm. high and 2.5 mm. wide with a 1 cm. light path were used in conjunction with a collimating cylindrical lens and a 2 x 12 mm. external slit. $E^{1\%,\ 1\ cm.}_{280}$ for thyroglobulin with this combination was 8.7.

The 103 µl. fractions were diluted with 200 µl. water and read against a blank of 5% sucrose at 280 mµ.

For estimation of protein at 210 mµ the fractions were diluted

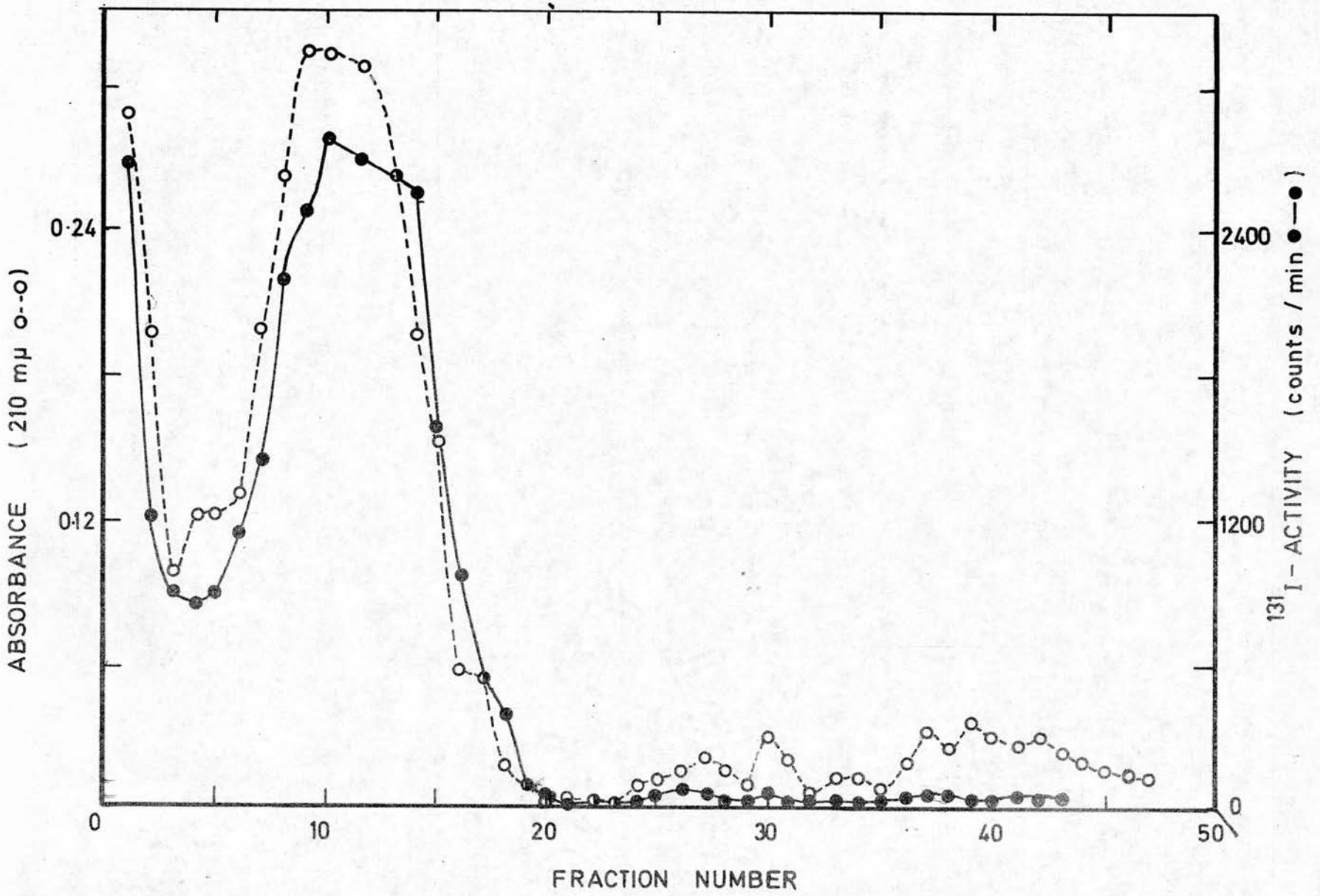


FIGURE 17 SUCROSE GRADIENT (5—20%) CENTRIFUGATION FOR 6 HOURS OF ^{131}I SHEEP THYROGLOBULIN.

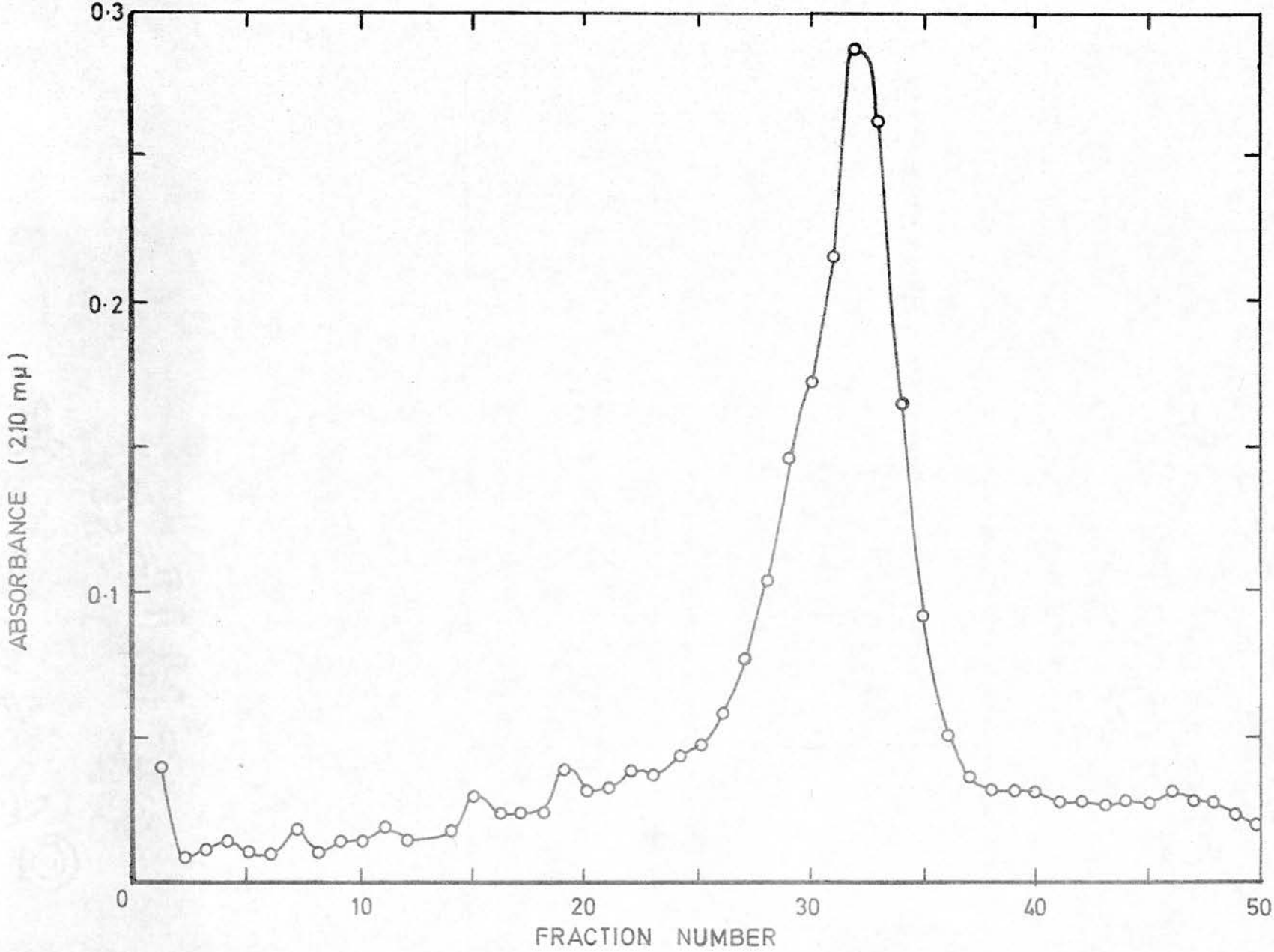


FIGURE 18 SUCROSE GRADIENT (5 – 20%) CENTRIFUGATION FOR 4 HRS OF SHEEP THYROGLOBULIN PURIFIED ON SEPHADEX G – 200.

to 3.30 ml. and read in 1 x 1 x 4 cm. cells. $E_{210}^{1\%,\ 1cm.}$ was taken at 207 (Salvatore et al., 1964).

The fractions were assayed for radioactivity, as described in Section V.1.1., after the second dilution.

III.4.5. Results: In the first experiment 0.75 mg. of ^{131}I-thyroglobulin in 100 µl. which had not been centrifuged was layered on to a sucrose gradient and centrifuged at the standard speed for 6 hr. A considerable quantity of material was found in the first two or three fractions from the gradient, i.e. some undissolved material had sedimented into the 20% sucrose pad at the foot of the tube. After 6 hr. the thyroglobulin began to overlap the insoluble material at the foot of the tube. Precentrifugation of the protein sample in later determinations almost entirely removed this heavy material.

Fig. 17 shows the absorption at 210 mµ and the radioactivity in each fraction. The amount of lighter radioactive contaminating material was negligible. This material absorbed relatively more strongly at 280 mµ than at 210 mµ and may correspond to material in the later fractions during elution of soluble proteins from G-200. The absorbance of the heavier fractions at 280 mµ was virtually the same as that at 210 mµ and has been omitted for clarity.

A sample of thyroglobulin (0.72 mg.) purified by filtration on G-200 Sephadex and precentrifuged was added to a 5-20% sucrose gradient and centrifuged at 40,025 rpm for 4 hr. The fractions were assayed for protein only at 210 mµ. The single peak, with maximum at fraction 32, contained almost all the material (Fig. 18).

A sample of 0.74 mg. ^{131}I-sheep thyroglobulin was centrifuged on a gradient. This also produced one peak with a maximum at fraction 32. The trailing edge of the peak appeared virtually

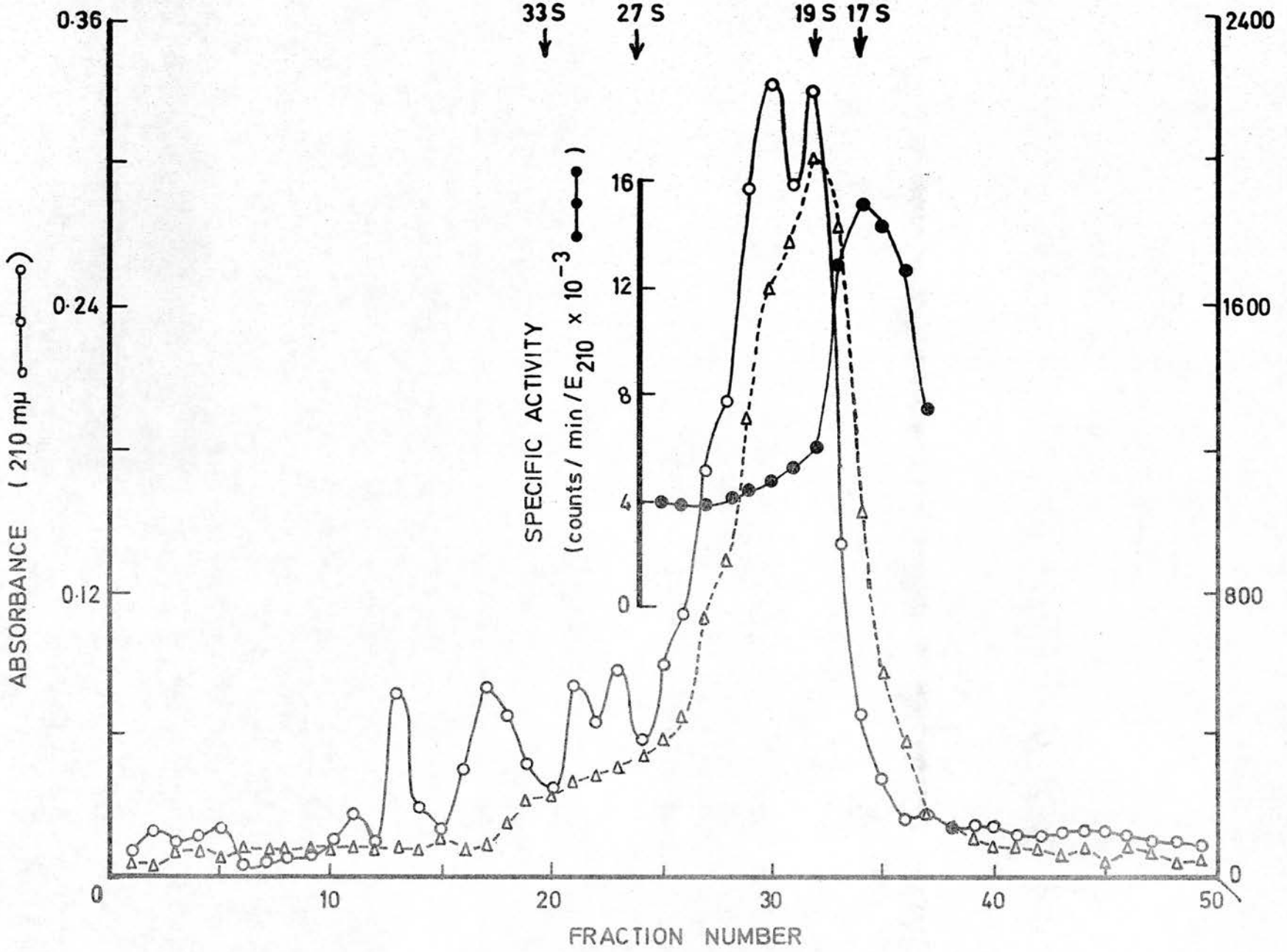


FIGURE 19 SUCROSE GRADIENT (5 – 20%) CENTRIFUGATION FOR 4 HR. OF ^{131}I– SHEEP THYROGLOBULIN.

uncontaminated. The leading edge contained some material slightly heavier than thyroglobulin. From Fig. 19 it can be seen that the lighter material in fractions 39-50 absorbing at 210 mμ was barely radioactive and as such was not a serious contaminant. Assuming that the main peak was composed of 19S protein by comparison with purified sheep thyroglobulin (Fig. 18) and that the rate of sedimentation was largely proportional to the Svedberg constants as the protein species had not yet attained equilibrium with the sucrose gradients, the radioactive material sedimenting just in front of the 19S peak had a range of S values from 33S to 27S and may have included the small amount of thyroglobulin dimer and trimer which accompany the 19S form.

The main peak of absorbance was bifurcated, an effect not shown by the radioactive assay. A natural irregularity in the gradient would have also affected the radioactive assay in the same way, assuming the specific activity of the protein was constant.

In fact, the specific activity of the ^{131}I-protein remained constant throughout the 210 mμ peak but rose sharply, almost four times, on the trailing edge of the protein. The point of highest specific activity corresponded to a sedimentation constant of 17S. As mentioned in the introduction, the newly-formed uniodinated thyroglobulin takes up iodine but remains in a immature state for some time before maturing to the main 19S form of thyroglobulin. This effect was also noted in the earlier sedimentation experiment (see Fig. 17) and during gel filtration (Fig. 11, following p. 69).

It appears feasible that the standard preparation of ^{131}I-thyroglobulin contains mainly 19S protein along with a little of the 17S, 27S and 33S forms. Although quantitatively there is little 17S present, its high specific activity will increase its contribution

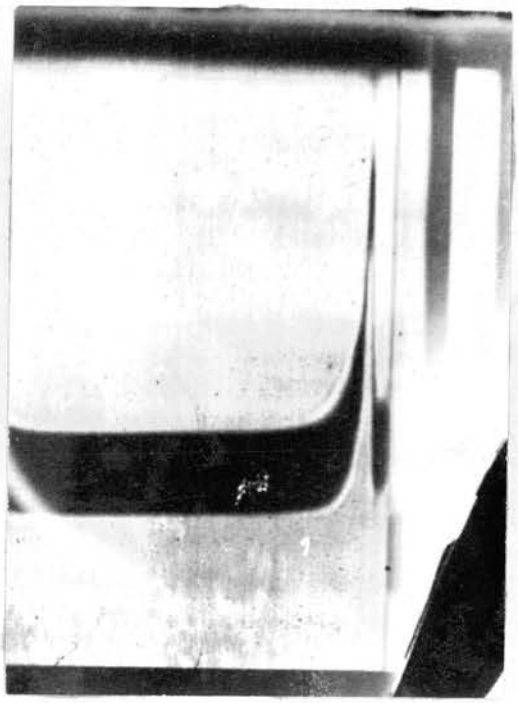


Plate 1

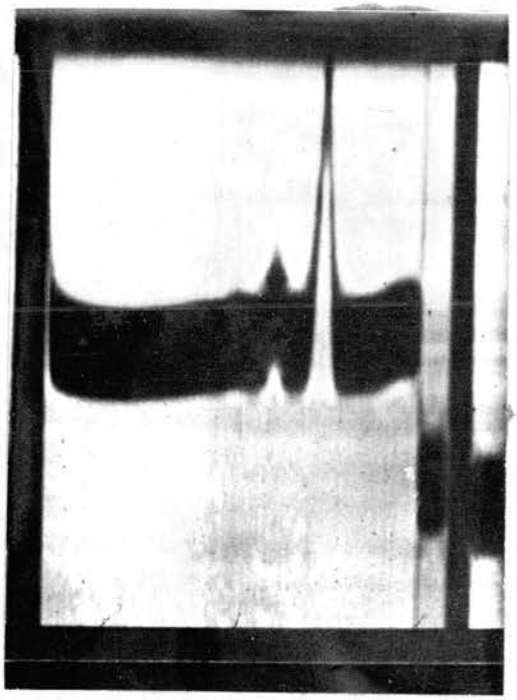


Plate 2

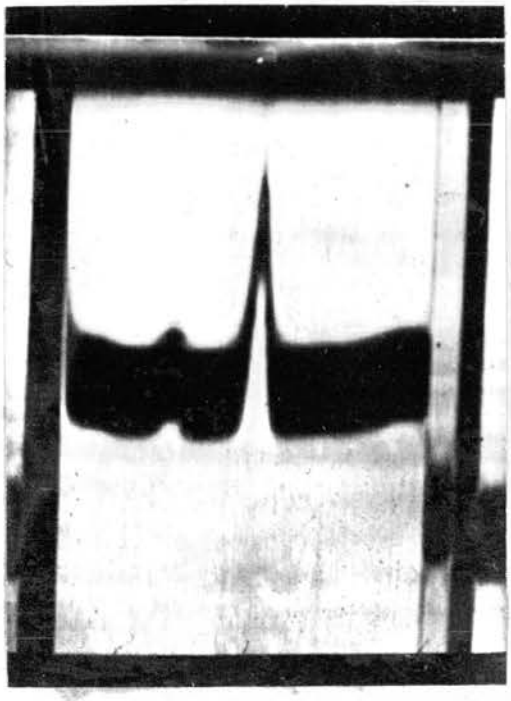


Plate 3

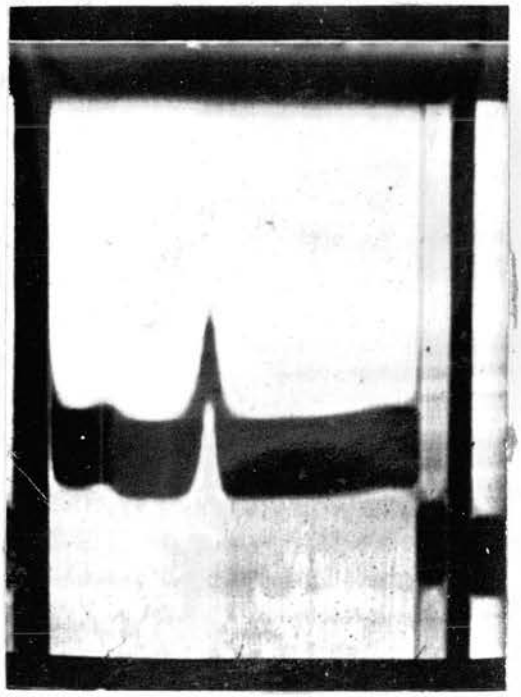


Plate 4

Plates 1-4. Schlieren patterns of sheep thyroglobulin (0.5% in 0.1M KCl:0.02M sodium phosphate, pH 7.4) centrifuged at 56,100 rpm for 30 min. Times of exposure after reaching maximum speed were 0.9 min. (Plate 1), 12.0 min. (Plate 2), 22.0 min. (Plate 3) and 27.8 min. (Plate 4). Sedimentation from left to right.

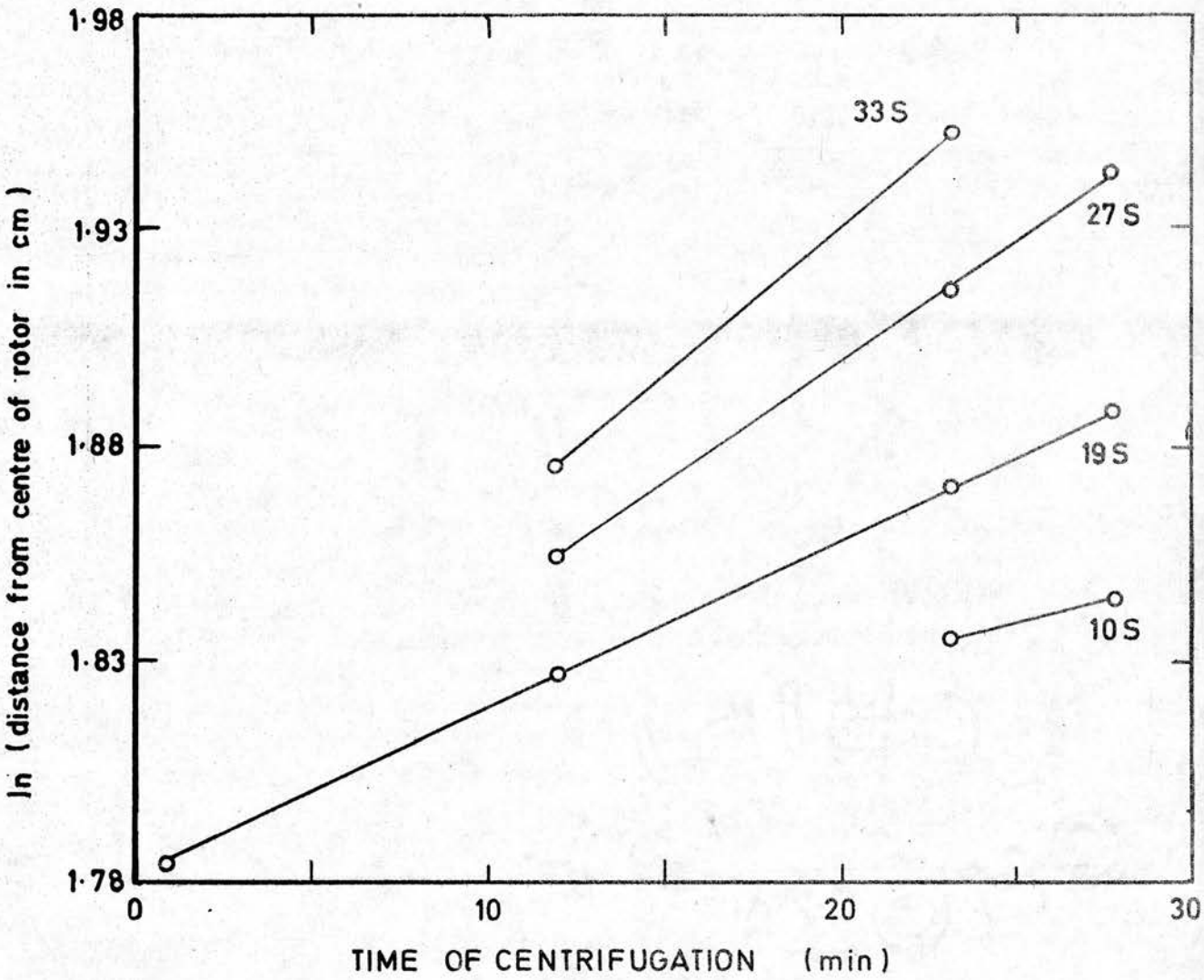


FIGURE 20 . MOVING BOUNDARY ULTRACENTRIFUGAL ANALYSIS OF STANDARD SHEEP THYROGLOBULIN.

in any assay, such as autoradiography, where radioactivity is the criterion.

This will make no difference to the enzymic hydrolysis as the thyroglobulin solution is heated to 80° for 5 min. before chymotryptic hydrolysis.

The two forms of the protein explain the bifurcation of the 210 mμ peak.

By the method of sucrose gradient centrifugation there was very little non-thyroglobulin contaminating protein.

III.5. Ultracentrifugation

III.5.1. Method: Sheep thyroglobulin which had been freeze-dried and stored at -20° for 60 dy. was dissolved in 0.1M KCl - 0.02M sodium phosphate buffer (pH 7.4) to a concentration of 0.5%.

The solution stood overnight at 4° and was centrifuged to remove any undissolved material.

The 0.5% solution was centrifuged in a Spinco Model E Analytical Ultracentrifuge at 56,100 rpm for 20 min. at an average temperature of 21.2°. The temperature of the rotor was taken before and after the run and the average value used to correct for the change of viscosity of water with temperature.

Photographs of the schlieren patterns were taken during the run with 10 sec. exposures and the odometer value noted each time after 5 sec. exposure. (Plates 1-4)

Fig. 20 shows plots of the natural logarithm of the distance of the protein boundary from the centre of the rotor against time for four protein peaks. The gradients of the best straight lines through the points for two of the peaks, found to be 19S and 27S protein, were computed by the method of linear regression.*

* For calculation of the sedimentation constants see Appendix I.

By substitution of the gradients in the Svedberg equation values for S were found. These were corrected to zero protein concentration at 20° by extrapolation of the increase in S value with decreasing concentration of protein (Malan, 1968) and by correction for the decrease in the viscosity of water with increasing temperature.

The standard error of the slope was also computed for the 19S peak.

The four visible protein bands had Svedberg constants, $S^{0\%}_{20,w}$ of 10.51, 19.15±0.35, 27.16 and 33.52.* Of these the last three values are those expected for thyroglobulin species. Both the 10S and 33.5S peaks were computed from two points and the 27S from three points.

To find the purity of the thyroglobulin sample the protein peaks were cut out from enlargements of plates 2 and 3, and weighed. Plate 3 had the best separation of the peaks. Of the total protein 76% was thyroglobulin and the thyroglobulin itself was 80.7% 19S, 13.8% 27S and 4.1% 33S. (Salvatore et al., 1965) found sheep thyroglobulin comprised 86% (mean) 19S protein and 11-17% (range) 27S protein and no 12S.

III.6. Amino acid analysis of thyroglobulin

If the standard thyroglobulin preparation (Section II.3.4., p. 53) was markedly contaminated with other proteins this would be reflected as an amino acid content at variance with that expected for purified thyroglobulin. Lissitsky (1966), after purifying saline extracts of sheep thyroid slices by ammonium sulphate fractionation and chromatography on DEAE-cellulose, made six separate determinations of the amino acid content. Comparison of

* For calculation of the sedimentation constants see Appendix I.

Table 31. Amino acid composition of sheep thyroglobulin

Results from (a) standard thyroglobulin preparation, (b) thyroglobulin purified on Sephadex G-200, (c) the average of (a) and (b), and (d) date of Lissitsky (1966).

Amino acid	Moles of amino acid/mole thyroglobulin			
	(a)	(b)	(c)	(d)
Asp	411	363	387	379
Thr	264	248	256	231
Ser	438	499	469	492
Glu	650	700	675	687
Pro	337	380	359	356
Gly	435	432	434	425
Ala	467	483	475	431
Val	321	315	318	322
Cys	221	-	221	203
Met	81	50	66	64
Ileu	120	121	121	134
Leu	494	475	482	502
Tyr	146	121	134	133
Phe	253	274	264	279
Lys	138	116	127	128
His	68	64	66	61
Arg	295	307	301	312

Correlation coefficients:- (a) vs. (d): 0.9900, (b) vs. (d): 0.9946, (c) vs. (d): 0.9956

these values for the amino acid content, with values determined for the standard thyroglobulin preparation, and for a preparation purified by gel filtration on Sephadex G-200, was expected to reveal how closely the standard thyroglobulin preparation resembled the purer preparations.

III.6.1. Methods: Standard thyroglobulin was purified on Sephadex G-200 (see Section III.2.2., p. 68): the fractions containing the central part of the thyroglobulin peak were pooled, dialysed to remove buffer and freeze-dried. Samples of the thyroglobulin preparations were dissolved in 200 volumes (w./v.) of constant boiling HCl, sealed in glass capillaries and heated at 105° for 18 hr. HCl was removed from the hydrolysate by alternate rotary evaporation and solution in water.

The final solution for analysis on an EEL Autoanalyzer was made up in 0.1N HCl and contained an estimated 0.25 μ mole leucine equivalent per ml.

The quantities of amino acids present were estimated from the areas of the peaks on the Analyzer chart record by comparison with standard values. These were corrected for the loss of labile amino acids and for the difference in specific extinction at 570 mμ. A final correction was made for the thyroglobulin carbohydrate, molecular weight 42,000 (Spiro and Spiro, 1965). Amino acid content was expressed as moles of amino acid per mole thyroglobulin.

III.6.2. Results: Table 31 shows the amino acid composition of the standard thyroglobulin preparation (A), of the Sephadex purified preparations (B), the average of these (C), and Lissitsky's (1966) data for comparison (D).

The correlation coefficients of A, B and C compared to D were found. From these the composition of the Sephadex purified

thyroglobulin was closer to the value found by Lissitsky than was the composition of the standard preparation. Part of the apparent difference in composition of the two preparations was due to variation in assay - the average value (C) being closer to Lissitsky's data than either of the separate results.

The use of correlation coefficients here may be open to the criticism that if the percentage of one amino acid in a contaminating protein is much lower and that of another is much higher substitution of the contaminating protein for thyroglobulin will have little effect on the significance of the correlation.

A more sensitive method for estimation of contaminating protein is consideration of the content of individual amino acids. Standard deviation for six determinations of each amino acid in thyroglobulin (as moles of amino acid per 660,000 g. of protein) were calculated from the data of Lissitsky (1966). The amino acids in the standard thyroglobulin preparation (Table 31, column d) differed little from the average of Lissitsky's (1966) values (Table 31, column d): 8 of the 17 amino acids were within one standard deviation, 13 of the 17 were within two standard deviations and the remainder within 5-10% of the average. Comparison of the amino acid content of thyroglobulin with that of 660,000 g. of a likely contaminating protein, such as serum albumin, revealed one amino acid within one standard deviation and a further 4 within two standard deviations, and 12 of the 17 amino acids differed by more than 10% from the average values for thyroglobulin.

Examination of the amino acids singly could be used to demonstrate small amounts of contaminating protein. For instance, the lysine content of serum albumin is 579 moles/660,000 g. of protein and of thyroglobulin is 128 ± 4.2 moles/660,000 g. of protein.

The percentage of serum albumin which could be detected, that is, yielding a mixed protein whose lysine content is greater than the average lysine value for thyroglobulin by the standard deviation, in this case is 0.85%.

This latter method would, if the amino acid content of contaminating proteins were known, provide a sensitive test of purity.

III.7. Analysis of ^{14}C-amino acids incorporated into thyroglobulin

Using a combination of electrophoresis and chromatography it proved possible to separate most of the amino acids in thyroglobulin. This separation was standardised by analysis of known mixtures of amino acids and applied to the separation of ^{14}C-amino acids from labelled thyroglobulin. From this was found the extent of labelling and interconversion of the amino acids during *in vitro* incubation with sliced sheep thyroids prior to incorporation into thyroglobulin. Further, once the specific activities of the individual amino acids were known the moles of a particular amino acid in a mixture could be calculated solely from a knowledge of its radioactivity. A carbon-14-labelled peptide, isolated by peptide mapping, was hydrolysed in acid and the resultant amino acids separated. By determining the radioactivity of each amino acid and knowing its specific activity in the parent thyroglobulin, the quantity of each amino acid in the peptide was found (Appendix III).

III.7.1. Methods and results: Known mixtures of amino acids were subjected to electrophoresis for 2 hr. in pyridine:acetic acid buffer (pH 6.5) at 1,800 v. The amino acids which were detected by very light spraying with ninhydrin, separated into four groups, namely the acidic amino acids, asparate and glutamate; histidine; the

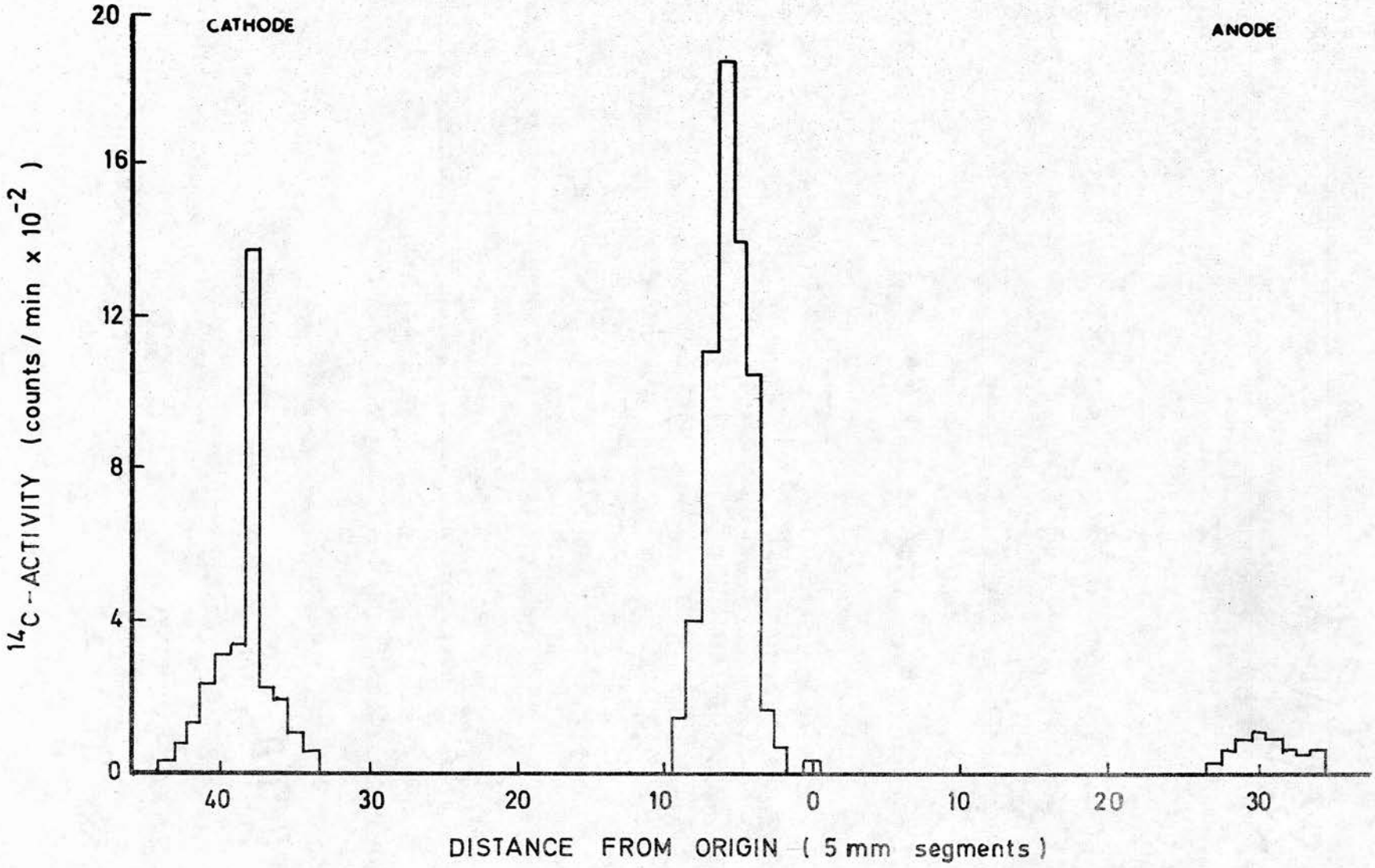


FIGURE 21a ELECTROPHORESIS AT pH 6·5 OF AN ACID HYDROLYSATE OF SHEEP THYROGLOBULIN LABELLED WITH ^{14}C - ARGININE, - LEUCINE AND -TYROSINE.

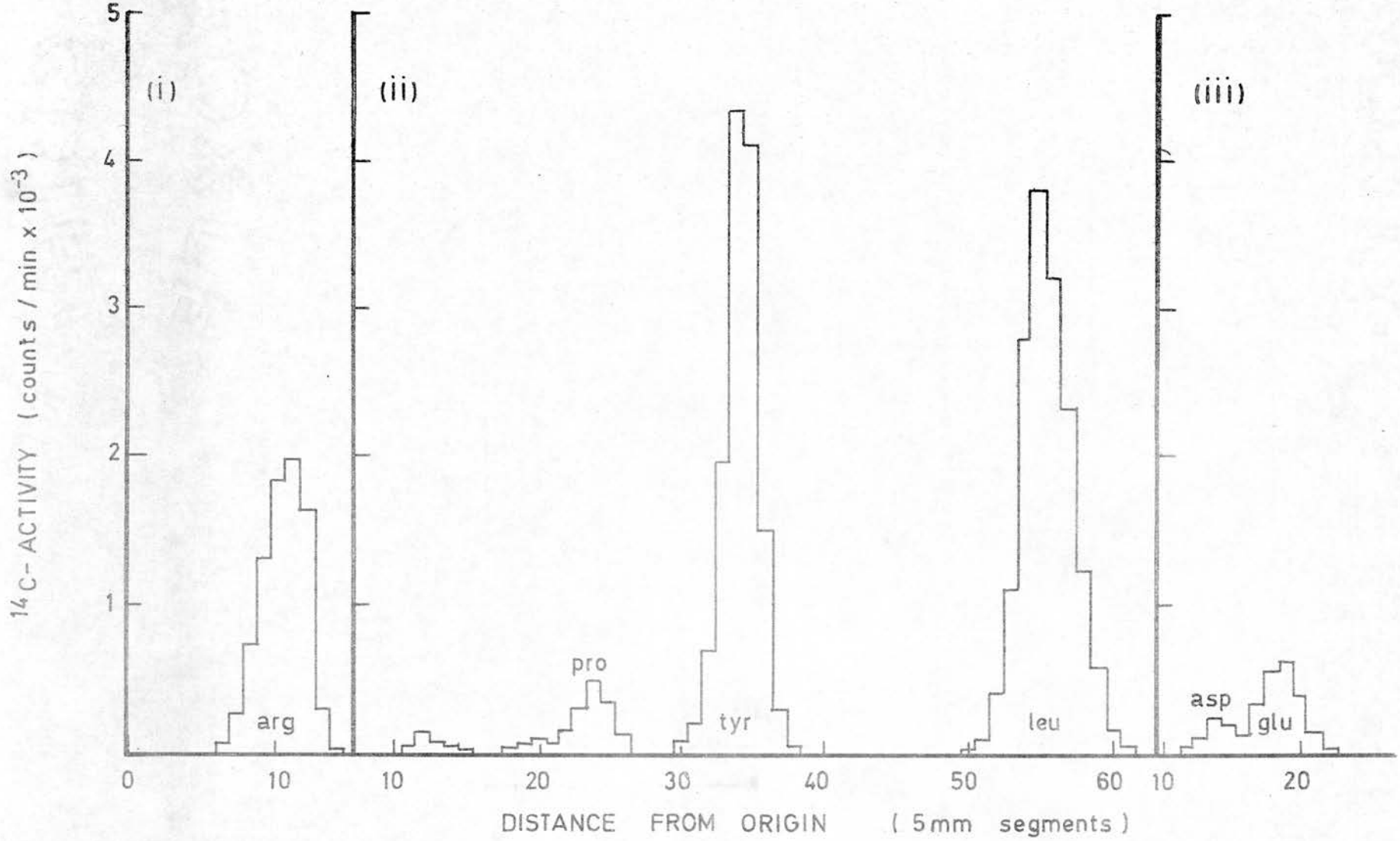


FIGURE 21 b FURTHER SEPARATION OF THE ^{14}C-AMINO ACIDS IN FIG. 21 a BY CHROMATOGRAPHY IN BUTANOL: ACETIC ACID – (i) BASIC, (ii) NEUTRAL. AND (iii) ACIDIC AMINO ACIDS.

Table 32. Interconversion of ^{14}C-amino acids by thyroid slices

Amino acid isolated	^{14}C-activity (count/min.)	No. of C-atoms	Moles amino acid/mole thyroglobulin	Absorbance at 510 mμ	Specific activities A*	B +
Arg	1,655	6	312	0.455	4.41	506
Asp	110	4	379	0.322	0.36	62
Glu	425	5	687	0.685	0.62	110
Leu	3,147	7	502	0.694	4.48	680
Tyr	2,642	9	133	0.080	11.03	3,000
Pro	283	5	356	0.370^{x}	0.51	150

* counts/5 min./C-atom/mole amino acid

\+ counts/min./E_{510}/C-atom

x estimated

Table 33. Partial separation in butanol : acetic acid of an acid hydrolysis of thyroglobulin labelled with ^{14}C-algal protein hydrolysate

Amino acids or pairs thereof	R_f values *	Specific activity counts/5 min./C-atom/moles amino acid
Leu/Ileu	0.500	0.463
Phe	0.455	0.460
Val/Met	0.347	0.418
Tyr	0.299	0.401
Pro	0.204	0.390
Ala/Thr	0.159	0.283
Ser/Gly	0.105	0.463
CySSCy	0.035	0.376
Arg	0.095	0.412
Lys	0.107	0.338
Glu	0.173	0.174
Asp	0.127	0.448

* average of three close values

neutral amino acids; and the basic amino acids, arginine and lysine. Strips containing the amino acids were cut out at right angles to the direction of electrophoresis. These strips had wicks cut in them and were developed in butanol:acetic acid for 20-24 hr. The longer chromatography, in which the solvent front ran off the paper, was possible because leucine has the largest R_f of 0.49 in this solvent. The acidic and basic amino acids separated completely. The neutral amino acids separated into individual acids or pairs thereof. Cystine, threonine, proline, tyrosine and phenylalanine separated and serine and glycine; valine and methionine; and leucine and isoleucine remained in pairs. If necessary, a further one stage separation would have differentiated between the amino acids in a pair.

Samples of thyroglobulin labelled with ^{14}C-arginine, leucine and tyrosine or with ^{14}C-algal hydrolysate were hydrolysed in acid, as detailed on p. 82 (Section III.6.1.), and the resultant amino acids separated into groups by electrophoresis. Rather than detect the amino acids by ninhydrin, which would lead to loss of $^{14}CO_2$, the electrophoresis area was counted in the chromatogram Scanner (Fig. 21:a) and suitable strips cut out for chromatography. The developed chromatograms were counted (Fig. 21:b) and the radioactive amino acids identified by their R_f values and finally by ninhydrin staining. The ninhydrin colour was stabilised by spraying with ethanolic cupric sulphate, eluted with methanol and its absorbance at 510 mμ found. Table 32 shows that as well as the three initially labelled amino acids from ^{14}C-arginine, -leucine, -tyrosine thyroglobulin there had been significant conversion of these to other amino acids which had also been incorporated.

Separation of the totally labelled amino acid mixture from

Table 34. Enzymic hydrolysis of ^{131}I-labelled sheep thyroglobulin

Enzyme, time of incubation and chromatography		^{131}I-activity of products of hydrolysis (%)								T_3/T_4	Ratio, R, of MIT/DIT
		MIT	DIT	I-tyrs	T_4	T_3	I-thyrs	I^-	Remainder		
Pancreatin (24hr.)	BA	47.7	25.5	-	-	-	5.85	3.16	17.6	-	1.87
	BDA	-	-	69.0	2.68	0.47	-	5.47	21.3	0.180	-
	BA	32.7	38.7	-	-	-	10.56	4.09	14.0	-	0.85
	BDA	-	-	65.0	3.86	0.30	-	4.33	26.4	0.078	-
	BA	48.3	25.0	-	-	-	7.00	7.00	19.8	-	1.94
	BDA	-	-	66.7	-	-	-	11.12	22.2	-	-
	BA	28.9	37.7	-	-	-	13.56	4.85	23.9	-	0.77
	BDA	-	-	66.3	2.36	-	-	5.43	25.9	-	-
Pancreatin (48 hr.)	BA	38.7	34.8	-	-	-	5.28	4.67	16.6	-	1.11
	BDA	-	-	79.5	0.53	1.15	-	4.91	14.0	2.17	-
	BA	46.2	37.1	-	-	-	5.68	3.28	7.8	-	1.25
	BDA	-	-	65.8	0.26	1.86	-	17.8	14.3	7.1	-
Pronase (24 hr.)	BA	42.5	45.4	-	-	-	4.29	1.85	6.0	-	0.94
	BDA	42.8	43.7	-	3.26	0.22	-	1.61	7.4	0.067	0.98
	BA	38.7	48.8	-	-	-	4.68	2.29	5.6	-	0.79
	BDA	40.8	45.3	-	4.32	0.25	-	1.87	7.7	0.058	0.90

thyroglobulin labelled with ^{14}C-algal protein was not complete, five neutral amino acids were separated and eight others were associated in pairs. The five charged amino acids were separated from all these. The activities of these peaks are listed in Table 33 and are corrected for the number of moles of each which are present in thyroglobulin, (Lissitsky, 1966) and for the number of carbon atoms in each. The unresolved pairs were corrected for the proportions of their constituent amino acids. The specific activities of the amino acids tended to very similar. The exceptions, such as the alanine/threonine pair and glutamate, could be explained as dilution of the alanine pool with 'cold' pyruvate and an active glutamate transaminase, respectively (Ragupathy et al., 1964).

Using a maximum of three separations, with marker amino acids, it will be possible to determine the amino acids in a peptide and, knowing the specific activity of each ^{14}C-amino acid in the original protein, it will be possible to find the ratios of the amino acids in a peptide. Since the peptides are small, it is probable that the lowest possible number of moles of amino acids per peptide will also be the true number. For application of this method to a peptide see Appendix III.

III.8. Iodoamino acid content of sheep thyroglobulin

The difference between the iodine metabolism of the thyroid in vivo and in vitro is generally reflected in a raised ratio of monoiodotyrosine and diiodotyrosine and a decreased content of iodothyronines in thyroglobulin. Lissitsky (1966) has found that sheep thyroglobulin contains nine molecules of monoiodotyrosine, six of diiodotyrosine and three of thyroxine per molecule of protein. The levels of labelled iodotyrosines and iodothyronines in sheep

thyroglobulin labelled with $^{131}I_2$ *in vitro* (Table 34) indicated that the production of iodotyrosines was little different from that *in vivo* but that very much less iodothyronine was synthesized *in vitro*.

III.8.1. Methods: Iodoamino acids were released from thyroglobulin by enzymic hydrolysis and separated by chromatography in two solvent systems. The methods used were basically those detailed on p. 106, Section V.1.5. for use with iodoamino acid peptides.

A sample of freeze-dried ^{131}I-thyroglobulin, usually 1 mg., was dissolved in 0.5 ml. of a buffer, pH 8.5, containing 0.07M Tris-HCl, 0.11M NaCl, 4 mM $MnSO_4$ and 0.04% TU (Tong and Chaikoff, 1958). To this was added 6 mg. Weddell pancreatin or 0.4 mg. pronase. Pancreatin hydrolysis was continued for 24 hr. or 48 hr. at 37° and hydrolysis with pronase for 24 hr. One drop of toluene was added to each hydrolysis to avoid bacterial action.

III.8.2. Results:

III.8.2.1. Hydrolysis of sheep thyroglobulin with pancreatin for 24 hr.: After 24 hr., chromatography of the hydrolysate showed that a considerable quantity of material was unhydrolysed, remaining on the origin; that all the radioactivity on the chromatograms was not included in one of the identified peaks; and that the iodotyrosine peaks were contaminated with partially hydrolysed material as reflected in the different monoiodotyrosine to diiodotyrosine ratios after duplicate hydrolyses of the same thyroglobulin preparation. In some hydrolysates the monoiodotyrosine to diiodotyrosine ratio was very high, approaching two instead of less than one. Thyroxine comprised approximately 3% and triiodothyronine, when detected, 0.4% of the total activity present. The unhydrolysed material amounted to over 20% of the total radioactivity. Deiodination amounted to 5%.

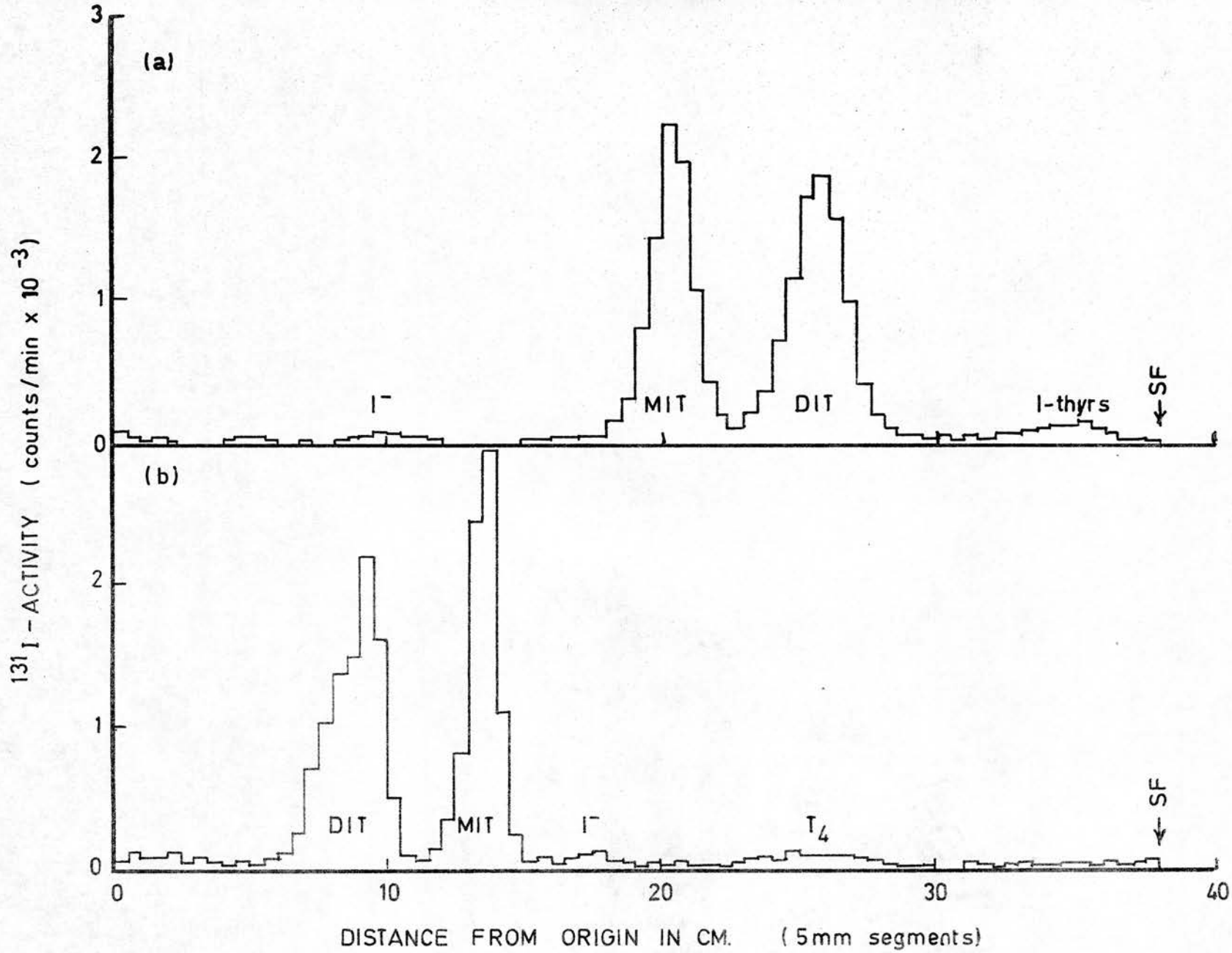


FIGURE 22 CHROMATOGRAPHY OF A PRONASE HYDROLYSATE OF ^{131}I - SHEEP THYROGLOBULIN IN (a) BUTANOL : ACETIC ACID AND (b) BUTANOL : DIOXANE : AMMONIA.

III.8.2.2. Hydrolysis of sheep thyroglobulin with pancreatin for 48 hr.: A further 24 hr. hydrolysis lowered the average monoiodotyrosine to diiodotyrosine ratio from 1.38 to 1.18. The contribution of unhydrolysed material fell to 12%. Although hydrolysis was improved, the content of thyroxine fell to 0.40% with a concomitant rise in the apparent content of triiodothyronine to 1.5%.

III.8.2.3.: Hydrolysis of rat thyroglobulin with pancreatin: Thyroglobulin, labelled *in vivo* by injecting a rat with 500 μC. of $Na^{131}I$ 8 hr. before sacrifice, and isolated by the standard method, was hydrolysed for 24 hr. with pancreatin. The pattern of labelling was quite different: the monoiodotyrosine to diiodotyrosine ratio was 0.49 and the preparation contained 8.71% thyroxine and 1.19% triiodothyronine. There was 9% deiodination and 11% of the material remained unhydrolysed.

III.8.2.4. Hydrolysis of sheep thyroglobulin with pronase for 24 hr.: The chromatograms of pronase hydrolyses were scanned using a mask so that each 0.5 cm. segment was counted. Very little unhydrolysed material was found and iodide was present to only 1.9%.

More diiodotyrosine was released by pronase hydrolysis and the ratio of monoiodotyrosine to diiodotyrosine fell to 0.90. This compares with a ratio of 0.75 for *in vivo* determinations (Lissitsky, 1966). The *in vitro* preparation contained 3.5% thyroxine and 0.24% triiodothyronine. Figs. 22:a and 22:b show the separation of the hydrolysate in BA and BDA. Table 34 contains the results of this, and a duplicate hydrolysis along with a summary of the results of pancreatin hydrolysis.

From the results the *in vitro* iodination in sheep thyroid slices appears to proceed to the iodotyrosine level similarily to the *in vivo* system. There is, however, little conversion of diiodotyrosine to

thyroxine. In similar preparations Ragupathy et al. (1964) were able to increase the thyroxine content of thyroglobulin to 15% only after treatment with thyroid stimulating hormone.

Chapter IV

PEPTIDE MAPPING OF THYROGLOBULIN

To isolate the sites of hormonogenesis in thyroglobulin it was necessary first to split the peptide chain by enzymic hydrolysis and then to separate the fragments.

This chapter deals with hydrolysis conditions, technique of peptide mapping, detection of separated peptides and distribution of radioisotopes in the peptides.

IV.1. General considerations

IV.1.1. Selection of hydrolytic enzyme: α-Chymotrypsin was used for the initial hydrolysis of thyroglobulin. This enzyme, with a pH optimum of 8.5 (Dixon and Webb, 1964) hydrolyses the peptide bond on the C-terminal side of aromatic residues. Chymotrypsin also attacks the bonds next to tryptophan, histidine and aliphatic amino acids, but at a slower rate.

If all the bonds next to aromatic residues are available for attack, each peptide will contain only one such residue. No more than one iodotyrosine, or iodothyronine, will be present in a peptide. The iodinated residues in thyroglobulin can then, in theory, be isolated.

Sheep thyroglobulin contains 279 phenylalanyl, 133 tyrosyl, 15 iodotyrosyl and 3 iodothyronyl residues per molecule (Lissitsky, 1966). On this basis alone, hydrolysis should release at least 431 peptides with an average molecular weight of approximately 1,500.

IV.1.2. Chymotryptic hydrolysis of thyroglobulin: It was necessary

to find a suitable length of time for chymotryptic hydrolysis, as the reaction was not expected to have a sharp end-point. Steric considerations, and the nature of adjoining amino acids may make the hydrolysis of some bonds slower than those next to aromatic residues.

Thyroglobulin prepared by the standard method still contained thyroidal proteases, peptidases and deiodinases. Heat denaturation was used to destroy their activities.

Both the length of α-chymotrypsin hydrolysis and the efficacy of the heat denaturation of the thyroidal enzymes were checked by autotitration under the conditions given in Section V.3.2.

The rate of addition of alkali to maintain the pH during hydrolysis revealed that the reaction was virtually complete in 5 hr. The standard time to ensure complete hydrolysis was 8 hr.

Hydrolysis of heat-denatured thyroglobulin released 704 free amino groups per mole of thyroglobulin. This figure rose to 1170 for the undenatured thyroglobulin. The difference between the hydrolyses was presumably caused by the activity of the peptidases in the freeze-dried undenatured thyroglobulin preparation.

IV.2. Methods for peptide mapping

IV.2.1. Standard conditions for the hydrolysis of thyroglobulin:

The freeze-dried thyroglobulin was dissolved in distilled water (1:150, w/v), heated in a water bath at $80\pm1^{\circ}$ for 5 min. and cooled. Ammonium bicarbonate, 3.2 mg./ml. and TU (to minimise deiodination), 0.4 mg./ml., were added. The pH of the solution was checked and, if necessary, adjusted to approximately pH 8.5 with gaseous ammonia. α-Chymotrypsin, 2% by weight of the thyroglobulin, was added and the hydrolysis continued for 8 hr. at 37° in a stoppered tube.

IV.2.2. Peptide mapping: Separation of the peptides from the chymotryptic hydrolysis was carried out by electrophoresis at pH 6.5 in pyridine:acetic acid: water (25:1:475) and by chromatography in butanol: 1 N-acetic acid (1:1) at right angles., (Malan, 1968).

The electrophoresis followed the free-hanging, white spirit-cooled method of Michl (1951). The apparatus consisted of a Shandon Chromatography Tank (30 x 30 x 12 in.) with a rectangular Perpsex trough (24 x 6 x 6 in.) supported 18 in. from the tank's base. The electrodes were platinum wires which entered the tank from the top and dipped into two volumes of buffer - one in the trough and the other in the foot of the tank. The tank was filled with white spirit to cool the paper during electrophoresis thus avoiding evaporation of the buffer. A cooling coil passing through the glass lid of the tank was immersed in the top of the white spirit.

Sheets of Whatman 3 MM paper 20 x 22.5 in. were supported vertically on a removable Perspex frame, fitted into the tank, allowing the ends of the paper to be immersed to about 1 in. in the two volumes of the buffer. By connecting the lower volume of buffer to the anode of a D.C. power pack, electroendosmosis acted against the siphoning effect of the paper. Neutral material moved 1-2 cm. to the cathode during a standard electrophoresis.

IV.2.3. Application of hydrolysate to paper: Weighed amounts of the freeze-dried chymotryptic hydrolysate were dissolved in the pyridine-acetate buffer and small volumes of this, usually 50μl., containing no more than 2 mg. of hydrolysate, were added to the dry paper from graduated pipettes. The area of the spot was kept small by evaporating the buffer with cold air from a hair dryer after each application. This led to a high density of material in a small area

and also to irreversible adsorption of material. The paper was then wetted with 60-70 ml. of buffer, hung on the Perspex frame and placed in the tank.

During electrophoresis the peptides tended to streak when the peptide map was overloaded. This restricted the quantity of material added to the paper to a maximum of 1.5 mg. To make detection of peptides easier and the time of exposure to X-ray film shorter, it was desirable that as much radioactive material as possible should be added to the paper.

It was found that the application of hydrolysate to a paper already wet with buffer enabled much larger quantities to be added in a much shorter time - often as little as 10 sec. instead of the former 10 min. The amount of material adsorbed on the paper was much smaller, as shown by the decreased radioactivity over the origin after autoradiography. Although the origin spot by the second method was often 0.5 in. in diameter, the peptide areas after peptide mapping were no larger than this and no larger than the smallest produced by adding hydrolysate to dry paper. Up to 2.8 mg. of material was added to peptide maps without signs of overloading.

In this way, the quantities of material on the peptide maps were increased with no decrease in resolution and with a slight decrease in time for the process.

IV.2.4. Conditions for peptide mapping: Electrophoresis was carried out at 1,800 v. for 3 hr. with a starting current of 33-37 mAmp, rising to 40-45 mAmp. at the end of the run.

After electrophoresis, the paper was dried at 40° for 2 hr. and trimmed. Wicks 2.5 in. in length were cut for chromatography at right angles to the direction of electrophoresis. Constant temperature (18°) chromatography tanks were saturated with the lower

phase of the solvent, and development of the chromatograms with the upper phase was continued for 11-12 hr. until the solvent front was 1.75 in. from the foot of the paper. The paper was dried at 40° for 3 hr. and trimmed to 17 in x 14 in. for autoradiography.

IV.2.5. Detection of peptides: The method for the detection of peptides produced during enzymic hydrolysis had to be sensitive and to provide clear resolution between closely-spaced peptides with similar mobilities during peptide mapping. No colour reagent available was sensitive enough and all such reagents would have been undesirable since they are bound to alter or combine with the amino acid residues.

A more sensitive method which did not affect the peptides was detection of carbon or iodine radioisotope labelling.

Two pieces of counting apparatus were considered for scanning the peptide maps - a BTL Argon Chromatogram Scanner with a gas-flow proportional counter and an Ekco Strip Counter, Type N679A, with 4π counting and a variety of speeds and apertures for increasing resolution. The BTL Scanner contains sets of counters which will scan simultaneously areas of 0.5 x 2.5 cm. or 1.0 x 2.5 cm. This gives inadequate resolution. The Ekco Strip Counter scans a strip 5 cm. wide continuously using different slit widths. Very slow scanning speeds are needed to give adequate resolution.

The large number of small peptides produced by hydrolysis could be resolved only by autoradiography of the peptide maps. The trimmed maps were stapled to 14 in. x 17 in. Iflex X-ray films in the dark and left for lengths of time dictated by the activity of the material on the paper. The autoradiograms were developed in Ilford D-154 and fixed in BJP-F HypoAlum Fixer. The darkened areas on the dry film were outlined with Indian ink and numbered.

Table 35. Sensitivity of Ilfex X-ray films to ^{14}C-activity

Films developed in Ilford D-154 and fixed with BJP-F Hypo-Alum Fixer

^{14}C-activity (mμC)	Time of exposure		
	1 day	1 week	2 weeks
0.1	-	+	++
1.0	+	++	+++
10.0	++	+++	++++
100	++++	+++++	+++++

+	just visible
++	faint but clear (adequate for detection)
+++	strong and clear
++++	nearly completely exposed
+++++	completely exposed

Films were exposed for different times to ^{14}C-leucine spotted on to Whatman 3MM paper which absorbed 89% of the weak β-radiation (average energy 0.155 MeV). From Table 35 the minimum activity needed for clear visualization after an exposure of 2 wk. was 0.25 mμC/sq. cm. Peptide spots were more diffuse than the standards and the practical minimum activity was 0.001 μC/peptide spot for an exposure of 2 wk. Peptide maps containing ^{131}I-material required in general exposures of the order of 1-2 dy. only.

For comparison the ^{14}C-leucine standards were counted on the BTL Argon Chromatogram Scanner and the Ekco Strip Counter, which were found to have lower resolution and lower efficiency respectively.

IV.2.6. Nomenclature of peptides on autoradiograms: The neutral peptides were denoted by N, those with excess of amino groups by A, and those which did not move off the origin during electrophoresis by O. ~~and~~ those with excess of carboxyls by C. Each peptide was given a numerical subscript and numbering was started at the origin. Consequently, peptides with the largest net charge and highest R_f had the largest subscripts.

This system of nomenclature was used on all peptide maps so that any one peptide will be found in approximately the same area on different peptide maps. However, a peptide from thyroglobulin labelled with $^{131}I^-$ is not necessarily identical with the peptide from thyroglobulin labelled with ^{14}C which bears the same alpha-numerical designation, though the two may be identical.

The code for the peptide maps contains 'PM' for peptide map: followed by the source of label, 'I', or (^{14}C-tyr)'; the hydrolytic enzyme, e.g. Ch. for α-chymotrypsin and the time of hydrolysis, usually 8 hr.

IV.2.7. Autoradiograms of peptide maps of doubly labelled thyroglobulin: Peptide mapping and autoradiography of iodine and carbon-14

labelled thyroglobulin revealed a large number of peptides. Superimposition of autoradiograms often showed ^{14}C-peptides overlapping ^{131}I-peptides. It was, however, necessary to show that these 'doubly-labelled' peptides were identical. This could most easily be achieved by showing the distribution of the two isotopes on a single peptide map made from doubly-labelled thyroglobulin.

The β^- emissions from carbon-14 have a maximum energy of 0.155 MeV with an average of 0.05 MeV. $^{131}I^-$ has maxima of 0.61 and 0.81 MeV with a mean value of 0.20 MeV. The large difference in energies enabled the selective absorption of the carbon-14 radiation with much smaller losses of $^{131}I^-$ radiation.

A spent X-ray film placed on a radioactive source decreased the emissions from carbon-14 to 0.17% and those from $^{131}I^-$ to 38%. Screening with aluminium foil or Whatman 3 MM paper did not exclude the carbon-14 and at the same time allow the transmission of a useful amount of ^{131}I-radiation.

Sheets of spent X-ray film were positioned between peptide maps of doubly-labelled thyroglobulin and unexposed X-ray films. After exposure, the films were developed and the peptide maps stored at -20° until the ^{131}I-activity had decreased to a negligible level. A second autoradiogram was taken of the carbon-14 distribution.

The two autoradiograms from the same peptide map were exactly superimposed and any doubly-labelled peptide was then identified.

IV.2.8. <u>Separation of neutral peptides from doubly-labelled thyroglobulin:</u> The neutral peptides were eluted from some of the peptide maps (for method, see Section V.1.3.) and rechromatographed in two dimensions in butanol: 1 N-acetic acid (1:1, v/v), BA, and butanol:

dioxane: 2N-ammonia (4:1:5, v/v), BDA. Autoradiograms were made of the radioiodine activity on these chromatograms which were then stored, like the peptide maps, until the iodine activity had decayed to a neglibible level, when autoradiograms of the ^{14}C-activities were made. This procedure further separated the peptides which were not separated by peptide mapping alone.

IV.3. Results

IV.3.1. Peptide maps of thyroglobulin labelled with ^{14}C-amino acids:

The sucess of peptide mapping depended largely on the specific activity of the labelled protein. Early peptide maps produced from thyroglobulin (specific activity 19.5 mμC/mg.) labelled with ^{14}C-tyrosine revealed only a few darkened areas after autoradiography for 1 mth. Most of the activity was associated with the neutral peptides, although a few low activity charged peptides could be distinguished.

Better peptide maps had to await an increase in the specific activity of thyroglobulin and this, coupled with an increased exposure time of 2 mth., revealed a wider distribution of activity.

A second series of peptide maps was made from thyroglobulin containing ^{14}C-tyrosine, or leucine or arginine, with specific activities of 78, 273 and 137 mμC/mg. respectively. Autoradiography of these peptide maps revealed approximately 30 peptides fairly equally divided in acidic, neutral and basic ones. Although the peptide maps had had almost the maximum quantity of hydrolysate added to them, the activity of the peptides was low. During electrophoresis the basic peptides had not separated well and the addition of larger quantities of hydrolysate would have led only to poorer separation.

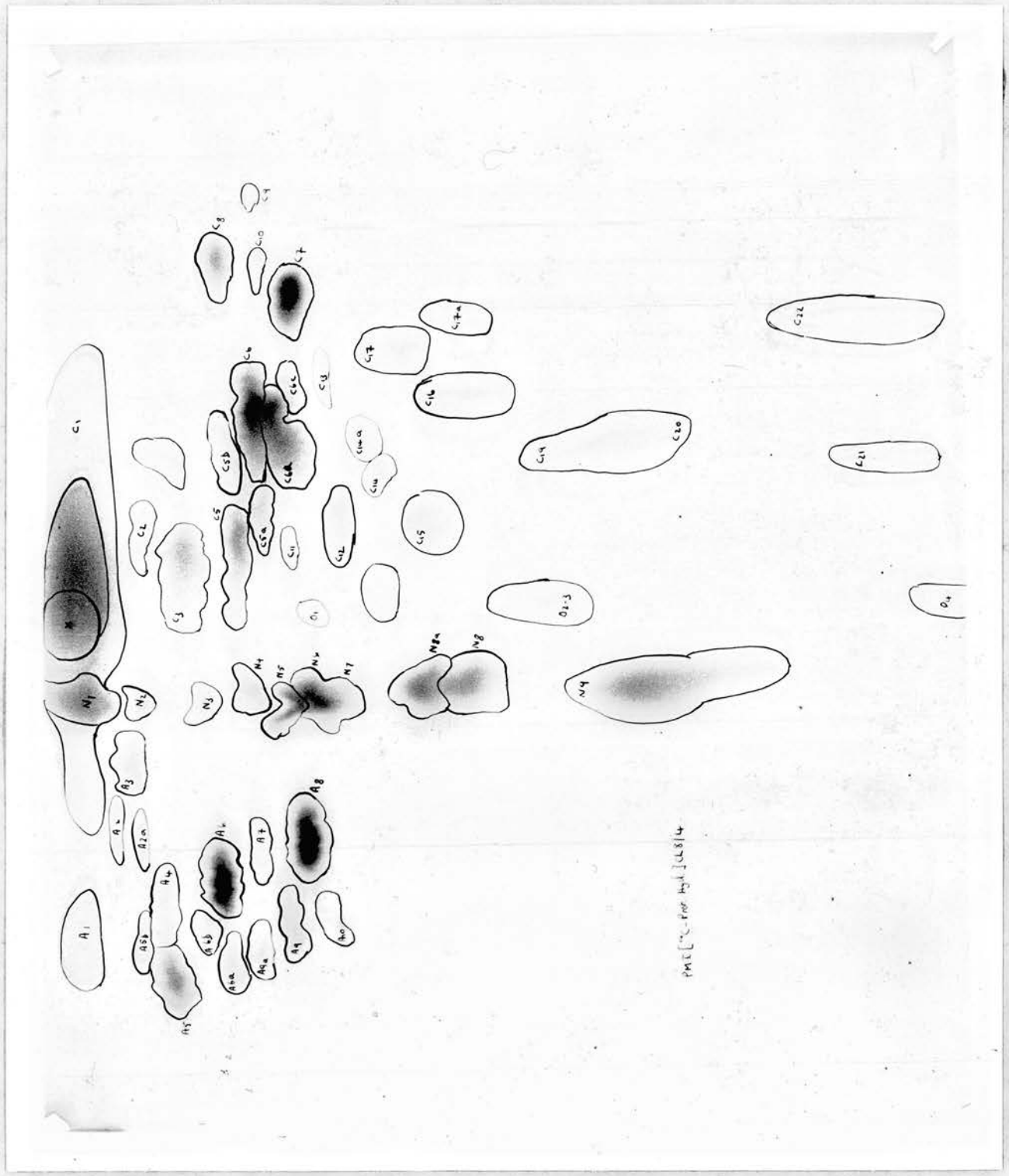


Plate 5. Autoradiogram of ^{131}I-activity on a peptide map of thyroglobulin labelled with $^{131}I_2$

Generally, more ^{14}C-arginine label was present in the basic peptides, as might have been expected. The peptides with higher R_f values in BA were more highly labelled with ^{14}C-leucine than with the other two amino acids.

Autoradiograms of duplicate peptide maps from the same preparations were quite superimposable with practically all the peptides matching, in spite of the low intensities of the spots and the poor resolution in the first dimension.

4 It was, however, difficult to tell whether peptides labelled with the different ^{14}C-amino acids were the same. Poor resolution combined with the large number of peptides ensured that slight variation in mobility and R_f values made positive identification of a peptide on two maps usually impossible.

As, however, several hundred peptides were produced by chymotryptic digestion, it was not surprising that few of the visible peptides corresponded, unless their higher activities indicated that the peptides might be larger and contain all the labelled amino acids.

IV.3.2. Peptide maps of thyroglobulin labelled with ^{131}I: The specific activities of these preparations were much higher than those of thyroglobulin labelled with ^{14}C-amino acids. Much less hydrolysate was added to the papers, resulting in small and more intense peptide spots (Plate 5). Relatively less of the radioactivity was present in the neutral amino acids. The number of peptides visible on any autoradiogram depended on the quantity of material added to the paper and on the length of exposure of the X-ray film.

Long exposure led to fogging of the film from the background radiation on the paper, and the sideways spread of energetic

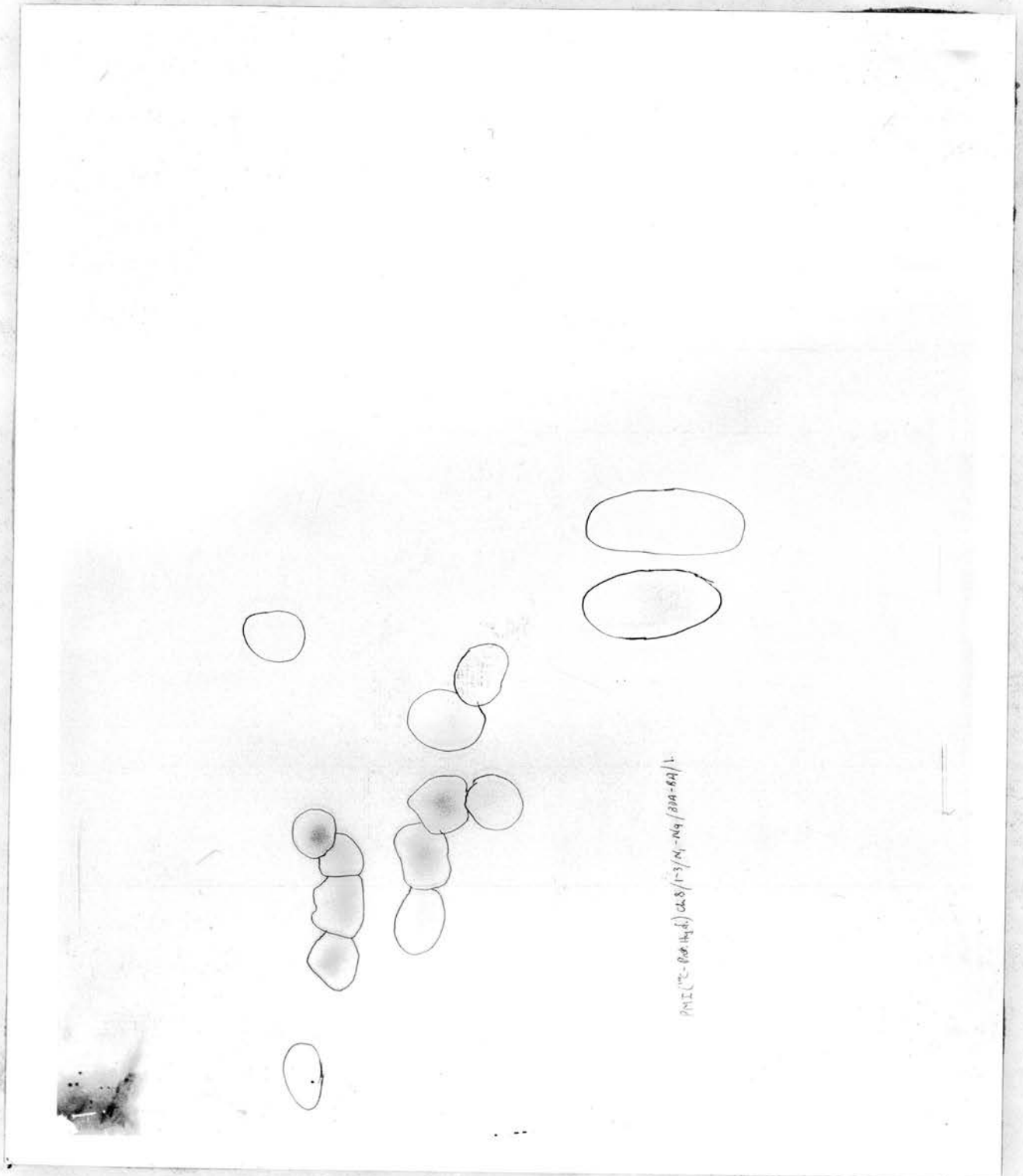


Plate 9a. Autoradiogram of ^{131}I-activity of neutral peptides on a two-dimensional chromatogram

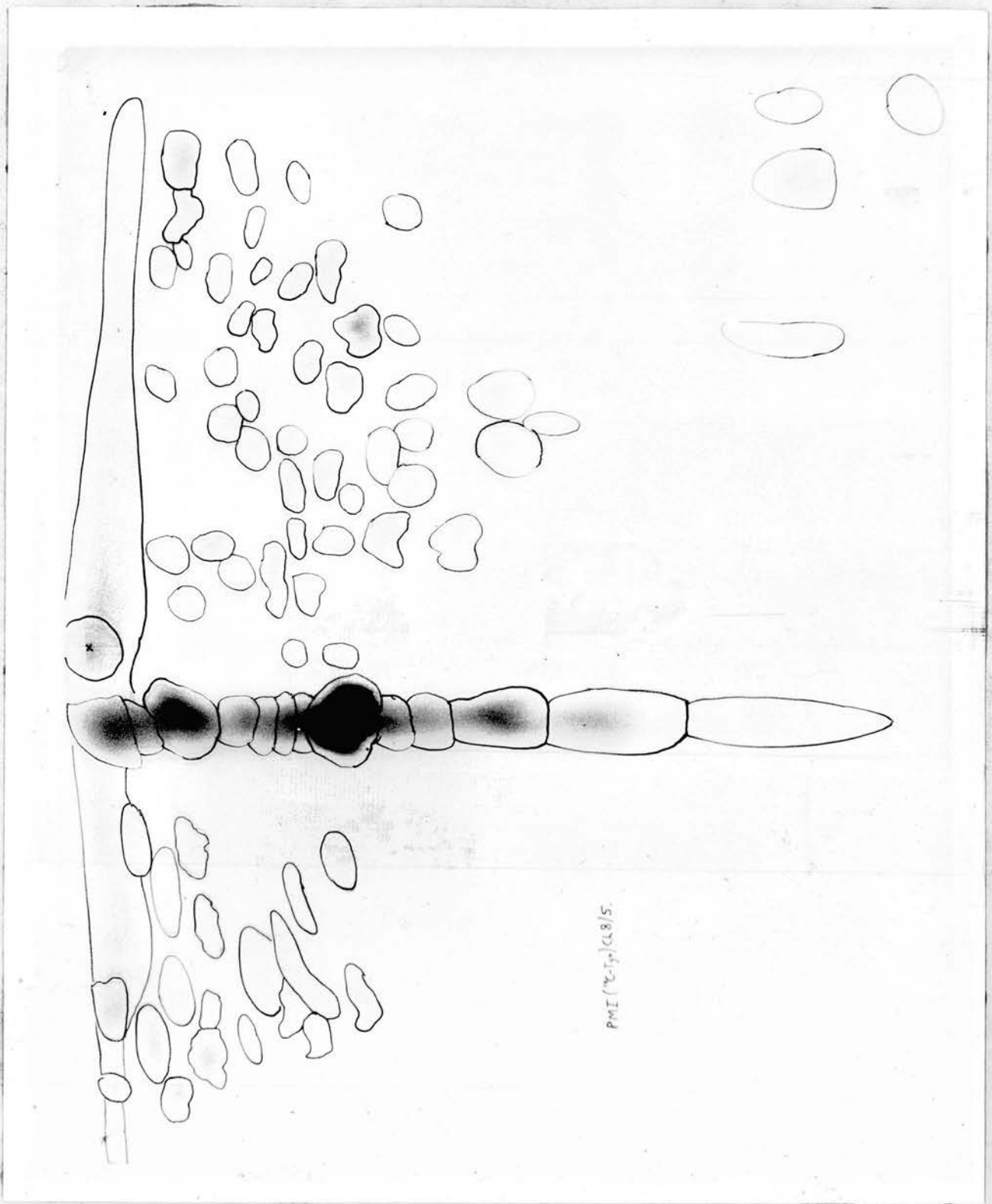


Plate 6. Autoradiogram of ^{14}C-activity on peptide map of thyroglobulin labelled with ^{14}C-tyrosine

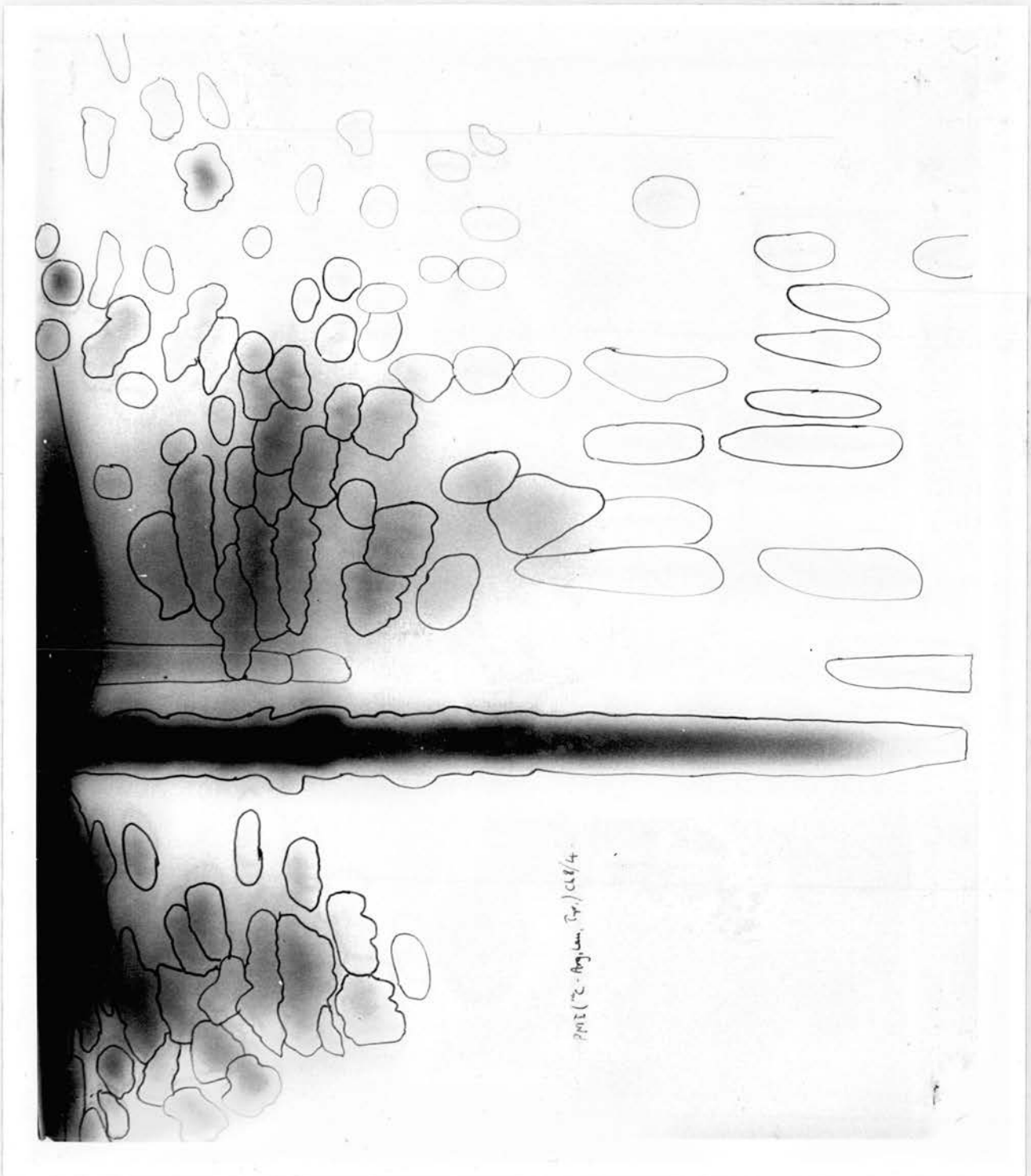


Plate 7. Autoradiogram of ^{14}C-activity on peptide map of thyroglobulin labelled with ^{14}C-arginine, -leucine, and -tyrosine

radiation through the paper blackening the film round the peptide proper.

The distribution of peptides in replicate chromatograms from the same chymotryptic digest and from chymotryptic digests of different preparations was highly reproducible. Under conditions of heavy load and long exposure, about 60-70 peptides became visible and could be positively identified on all the peptide maps of iodine-labelled sheep thyroglobulin. A dozen of these peptides which were intensely labelled and apparently uncontaminated were chosen for further study.

IV.3.3. Peptide maps of doubly labelled thyroglobulin:

IV.3.3.1. Autoradiograms of radioiodine activity: The iodoamino acid peptides from different preparations were clearly and reproducibly separated on the peptide maps. Two-dimensional chromatography of the neutral peptides separated N_6, N_7, N_8, N_9 and O_3 from 12 less active peptides which had the same R_f values as these peptides in BA but different R_f values in BDA (Plate 9,a).

IV.3.3.2. Autoradiograms of the carbon-14 activities of the series PMI(^{14}C-tyr)Ch8 - (Plate 6): The peptides labelled with ^{14}C-tyrosine had separated clearly into 41 acid, 19 basic and 12 neutral. The number of the latter peptides, after two-dimensional chromatography, increased to 31 two of which still contained most of the activity.

A total of 91 peptides were detected at this specific activity. Sheep thyroglobulin contains 133 tyrosine residues per molecule and the majority of these appeared to be labelled to a significant extent.

IV.3.3.3. Series PMI(^{14}C-arg,leu,tyr)Ch8 - (Plate 7): The autoradiograms revealed approximately 70 acid, 26 basic and, after two-dimensional chromatography, 40 neutral peptides - a total of 136.

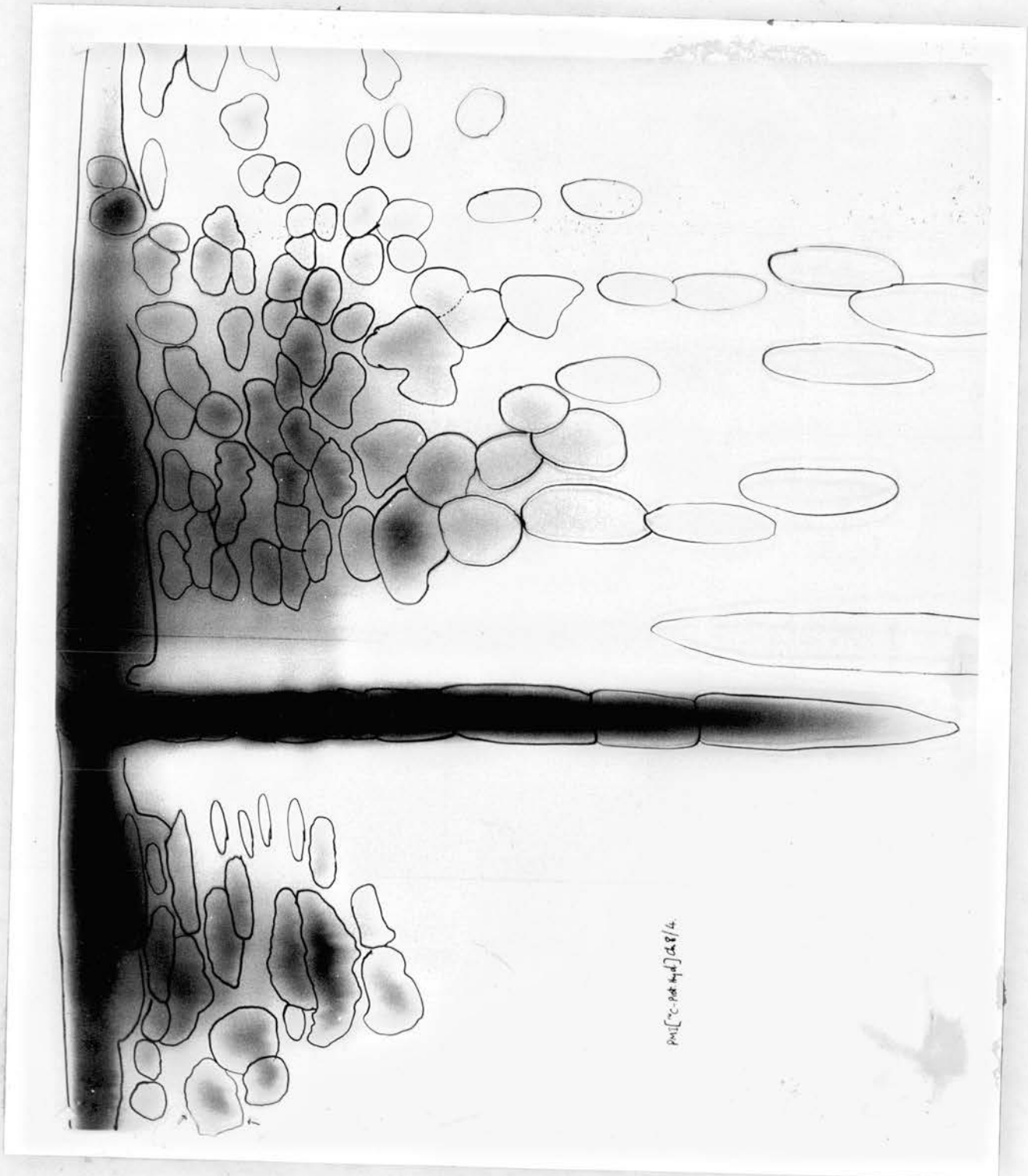


Plate 8. Autoradiogram of ^{14}C-activity on peptide map of thyroglobulin labelled with ^{14}C-protein hydrolysate

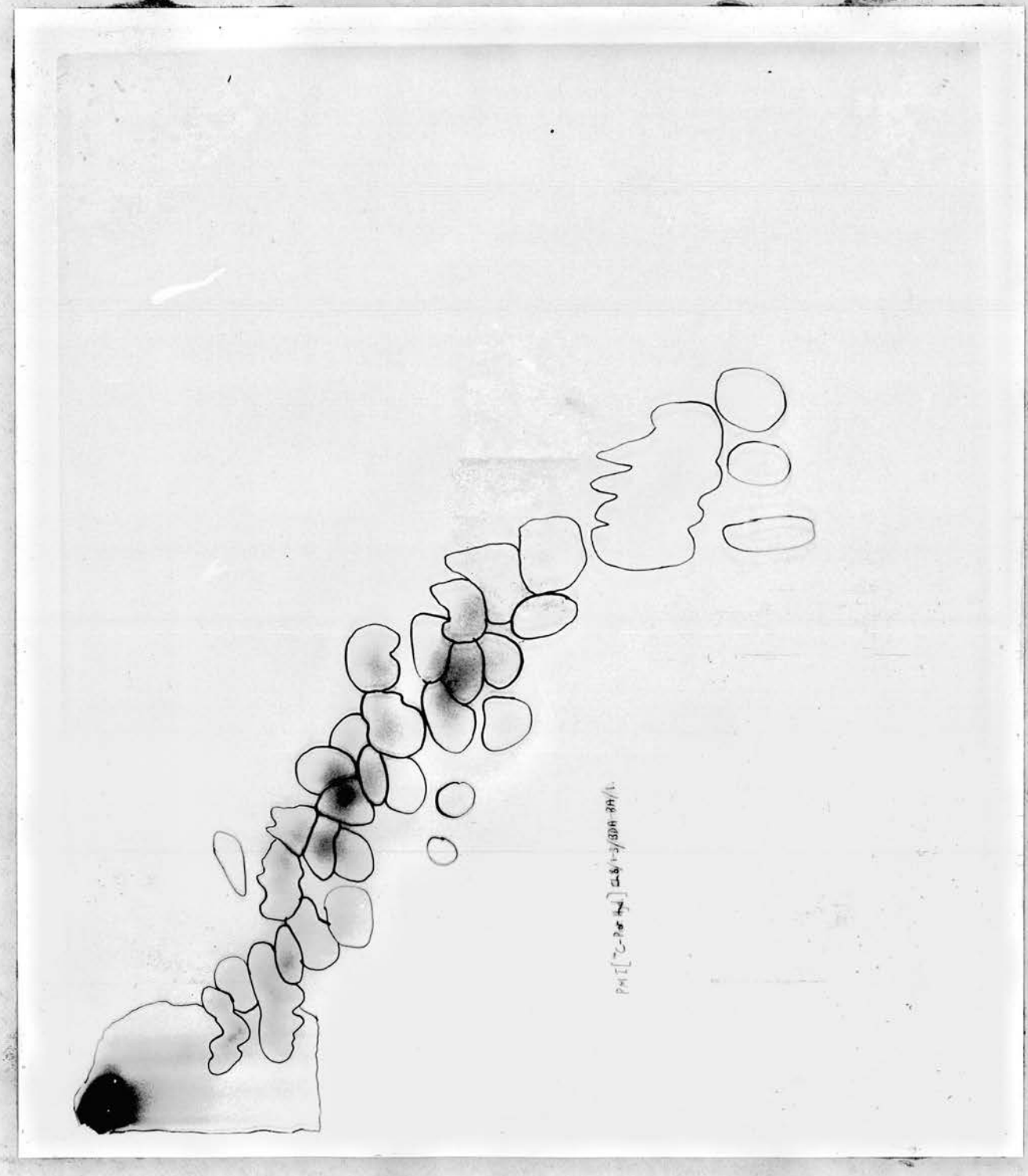


Plate 9b. Autoradiogram of ^{14}C-activity of neutral peptides on a two-dimensional chromatogram

IV.3.3.4. Series PMI(^{14}C-prot.hyd)Ch8 - Plate 8: The ^{14}C-amino acids from the algal protein hydrolysate labelled 84 acid, 25 basic and, after two-dimensional chromatography, 34 neutral peptides (Plate 9,b) - a total of 143. Plates 5 and 8 are the autoradiograms of the ^{131}I- and ^{14}C-activities of the same peptide map.

IV.4. Comparison of the autoradiograms from the doubly labelled thyroglobulin

The pairs of autoradiograms from each peptide map were exactly aligned with each other by the holes left by stapling the chromatograms to the unexposed films. As criteria for two spots to be produced by radiation from the same peptide the darkened areas on the two films must superimpose exactly and the outline and shape of the spots must be the same.

Peptides which appeared to have contained both radioiodine and carbon-14 label were classed as either likely to be the same, or possibly the same, or those whose overlap appeared to be coincidental.

Very few of the ^{14}C-tyrosine labelled peptides contained any radioiodine. This was partly due to the low activity of the peptides which decreased both the number of peptides detected ~~present~~, and also made it more difficult to outline their exact shapes. Even the most active radioiodine-containing peptides were no better than possible matches.

The additional labelling with three amino acids increased both the clarity and the number of the darkened areas. Some peptides were thought, with a fair amount of surety, to contain both radionuclides.

Labelling from the algal hydrolysate again increased the

radioactivity of the peptides; again only a few peptides appeared on both autoradiograms.

Although the ^{14}C-tyrosine peptides were faint, over 50% of these matched the peptides on the other ^{14}C-labelled autoradiograms. The correspondence between the more highly labelled preparations was greater.

Thus many of the faint ^{14}C-tyrosine peptides have their activities reinforced by labelling of the other amino acid residues in the peptide. Even then, these highly labelled and distinctive peptides do not correspond to peptides containing significant levels of radioiodine except possibly in a few cases.

IV.5. Conclusion

The distribution of radioiodine labelling among the peptides appears to strengthen the hypothesis that only a restricted number of the tyrosine residues in thyroglobulin can be iodinated and that of these iodinated residues, only a relatively small number are intensely labelled. It has been mentioned earlier (p. 19) that probably only 40% of the 133 tyrosine residues in sheep thyroglobulin are available for iodination. On the assumption that each of the iodine-labelled peptides contains only one iodotyrosine residue (an assumption later shown to be substantially valid, p.109) the number of iodopeptides found, between 60 and 70, is close to the number expected, about 50. Some of the iodopeptides contained very little radioiodine and may have resulted from the slow chymotryptic hydrolysis of less reactive peptide bonds. If this had happened the actual number of iodinated tyrosines was less than the number of iodopeptides and closer to the expected value.

The number of intensely labelled iodopeptides, about 12,

agrees closely with the 15 molecules of iodotyrosine per molecule of sheep thyroglobulin found by Lissitsky (1966). The thyroglobulin from which the peptide maps were prepared was iodinated *in vitro* and contained little iodothyronine.

Although the ^{14}C-tyrosine label in the sheep thyroglobulin was distributed among a large number of peptides very few of these were also labelled with radioiodine. This result confirms the finding of Mauchamp *et al.* (1965) who showed that thyroglobulin from sheep slices incubated with $^{125}I^-$ and ^{14}C-tyrosine contained no ^{14}C-label in the iodotyrosines. The two labels may well not be present in the same thyroglobulin molecules but they should be present in molecules which will yield peptides which are similar except for the accident of labelling.

Experimentally it had been found that the ^{131}I-thyroglobulin was heterogeneous: lighter material (e.g. fractions 33-37 of Fig. 19, following p. 78) containing a higher level of radioiodine than the bulk of the protein. If the recently synthesized lighter thyroglobulin molecules yield peptide maps differing from those obtained from older thyroglobulin this may provide an explanation for the small amount of ^{14}C-tyrosine in the iodotyrosine peptides.

Chapter V

PURIFICATION AND INVESTIGATION OF PEPTIDES

The last chapter detailed the production and separation of the labelled α-chymotryptic peptides from thyroglobulin. The iodoamino acid peptides contain those parts of the thyroglobulin molecule which had undergone iodination and consequently where synthesis of the iodothyronines would occur under suitable conditions.

Further investigation of these peptides was dependent on their being pure and of a small size. Although α-chymotrypsin was expected to separate all the iodoamino acid residues from one another large peptides might contain more than one of these. Once the peptides had been reduced to a suitable degree of purity and size to contain only one iodinated residue, each individual site of iodination could be studied.

Although the formation of diiodotyrosyl residues by the further iodination of monoiodotyrosyl residues seems well established it is not clear what conditions are necessary for this conversion or for the initial iodination of the tyrosyl residue. Could anything of the iodination mechanism be learned from the degree of iodination of each tyrosyl residue as represented by the absolute amount of attached radioiodine? If the access of iodine to certain tyrosyl residues is sterically restricted this will presumably result in the formation of small proportions of mainly the monoiodinated forms of these residues. Tyrosyls more exposed to iodine will be increasingly converted to their diiodinated forms.

As well as limiting access of iodine, steric effects will

become noticeable where the entry of one iodine atom into a tyrosyl ring is followed by configurational changes, such as rotation about the bond para to the phenolic hydroxyl group, precluding the addition of a second iodine atom. A tyrosyl residue of this type could be completely monoiodinated and might contain a level of radioiodine higher than half that in the diiodinated form of residues with restricted access to iodine.

As well as determining the total radioiodine contents of peptides after 8 and 24 hr. when the ^{127}I- and ^{131}I-isotopes had largely equilibrated, investigation of the changes in labelling of some of the most intensely labelled peptides after shorter times might allow the identification of the mono- and diiodinated forms of a single tyrosyl residue. The radioactivities of the two forms, isolated as peptides with similar mobilities, were expected to show a product-precursor relationship.

V.1. Methods

V.1.1. Counting of peptides: Radioactive peptides were detected as darkened areas on X-ray films. These were outlined and identified as described above (pp. 94-5). The outlines of the spots were traced with pencil on to the original peptide map. Before any further treatment, the peptide spots were cut out, placed in annular polythene cups and counted by scintillation; the cups fitting over an aluminium-sheathed NaI-lithium crystal linked to an Ekco Automatic Scaler (type No. N610B), which incorporated a pulse height analyser. The discrimator voltage (pulse height voltage) and gate width were set to give maximum counts with a background of approximately 2 counts/sec.

The areas of peptide map containing the peptides were

irregular in shape, but in the majority of cases most of the activity was concentrated in a small part of the spot. For this reason, the paper spots were folded along their major axis, the folds bisecting the areas of highest activity. The papers were placed in annular cups so that the folded edges were parallel to the base of the cups. As more scintillations are produced at the base of the crystal, the above procedure improved the geometry of counting and a large source of error was minimised.

V.1.2. Efficiency of counting procedure: Duplicate samples of $^{131}I^-$ were pipetted on to squares of Whatman 3MM paper, allowed to dry and the paper folded through the spot. The activities, counted as above, differed by less than the standard deviation of the counts due to the randomness of emission. The efficiency of counting under these conditions was 6.5%.

V.1.3. Elution of peptides: The peptide spots were cut with scissors into pieces approximately 1 mm. square. Depending on the quantity of paper these were added to 15-30 ml. volumes of ethanol:2N-ammonia (1:1, v/v) containing 0.04% TU to decrease deiodination. A Silverson homogenizer was used to pulp the paper. The pulp was then transferred with washing to a sintered funnel and the solvent filtered off by suction. The paper fibre was resuspended in a little solvent with a stirring rod, the solvent filtered off, and the residue washed by suction with a little more solvent.

The combined filtrates were taken almost to dryness on a rotary evaporator, with its water bath kept below 40°, before being stored at -20°.

To check the efficiency of elution, the dried paper residues were transferred to annular polythene cups and counted. A typical series of elutions from 2PMICh8/1 ranged from 99.75% to 99.93%.

Extractions were usually greater than 99%.

V.1.4. Hydrolysis of peptides: Conditions of hydrolysis were the same as for the hydrolysis of thyroglobulin. The dry peptide material was taken up in 0.5 ml. buffer and 6 mg. pancreatin or 0.4 mg. pronase were added. Hydrolysis was continued for up to 48 hr., at 37°, with pancreatin and for shorter times, as given in the text, when pronase was used.

V.1.5. Separation and detection of products of hydrolysis: After hydrolysis 0.1 ml. of concentrated ammonia was added to the hydrolysate to stop the enzymic reaction and to ensure that the iodoamino acids were soluble.

Separation (Plaskett, 1964) was carried out at a temperature of 18° on 5 x 17 cm. strips of Whatman 3MM paper with 5 cm. wicks cut at one end to slow down the rate of descent of the solvent front to approximately 17 hr.

Samples of the hydrolysate were added, as 2.5 cm. streaks, to the paper strips, 2.5 cm. beyond the wicks. Some chromatograms were spotted with small quantities of iodide, monoiodotyrosine, diiodotyrosine, thyroxine or triiodothyronine in ethanol:2N-ammonia (1:1, v/v) as standards. Duplicate chromatograms were developed in butanol:1N-acetic acid (1:1, v/v), BA, and butanol:dioxane:ammonia (4:1:5, v/v), BDA, overnight and dried for 2 hr. at 40°.

Before counting, the paper strips were attached at the ends by Sellotape to Perspex covers, marked in 1 cm. segments on metal slides. These were slid into the BTL Chromatogram Scanner and counted for suitable times. The 31 counting heads each counted an area nearly 1 cm. along the strip and approximately 2.5 cm. across the paper. For more precise work a mask was fitted over the paper which allowed areas of 0.5 x 2.5 cm. to be counted, thus doubling

the resolution with a loss of only 10% of the initial activity. Histograms were drawn of the activity in each segment and, from this, the compounds were tentatively identified. The activity on each chromatogram was summed and the percentage activity in each fraction calculated.

The identities of the radioactive peaks were confirmed by spraying the papers on which standards had been run with reagents specific for iodide, iodotyrosines and iodothyronines. During spraying with a reagent specific for one standard the other areas were covered in turn with filter paper.

Iodide sprayed lightly with 1% aqueous palladium chloride appeared as a dark brown spot (Gross and Leblond, 1951). Iodotyrosines and iodothyronines were sprayed with diazotized sulphanilic acid prepared immediately before use by mixing equal volumes of ice-cold 0.05M sulphanilic acid in 9% HCl and 4.5% aqueous $NaNO_2$. After air drying, a second spray with 1N-sodium carbonate or exposure to ammonia vapour revealed the iodoamino acids as orange/yellow areas. This stain is specific for iodoamino acids, but when these were the only compounds on the chromatograms 0.1% ninhydrin in butanol:acetone (1:9, v:v) was used to stain the amino groups. This colour reaction was made permanent by spraying with a saturated solution of hydrated cupric sulphate in ethanol. The purple ninhydrin colour became pink on a very pale green background.

Chromatography with BA separates the iodotyrosines from each other, from the iodide and from iodothyronines. The latter separate in BDA, although the iodotyrosines seldom do. Table 36 contains the R_f values in the two systems (see Figs. 22,a and 22,b, following p. 88).

In general, the chromatograms were not stained, since the radio-

Table 36. R_f values for the iodoamino acids chromatographed in butanol:acetic acid and butanol:dioxane:ammonia

Butanol:1N-acetic acid (1:1, v:v)				Butanol:dioxane:ammonia (4:1:5, v:v:v)			
MIT	DIT	I-thyrs	I^-	I-tyrs	T_4	T_3	I^-
0.61	0.75	0.96	0.28	19 - 27	0.68	0.80	0.46
0.59	0.75	0.93	0.25	14 - 25	0.65	0.77	0.42
0.49	0.56	0.92	0.28	23 - 30	0.55	0.76	0.46
0.62	0.63	0.92	0.26	22 - 27	0.69	0.66	0.55
0.62	0.74	0.95	0.29	11 - 19	0.73	0.81	0.45
0.64	0.78	0.97	0.24	22 - 30	0.68	0.78	0.44
0.45	0.80	0.91	0.28	25 - 35	0.52	0.71	0.51
0.51	0.67	-	0.25	20 - 29	0.67	0.81	-
0.57 *	0.71 *	0.94 *	0.27 *	0.20 - 0.28 +	0.63 *	0.76 *	0.47 *

* mean + mean range

Table 37. Hydrolysis of iodoamino acid peptides with pancreatin

Overt unhydrolysed material is included in the 'Remainder' fraction; otherwise it is evident as contamination of peaks leading to discrepancies between the values of a fraction in the two solvent systems.

Peptide	Solvent	^{131}I-activity of products of hydrolysis (%)								T_3/T_4	Ratio, R, of MIT/DIT
		MIT	DIT	I-tyrs	T_4	T_3	I-thyrs	I^-	Remainder		
2PMICh8/	BA	85.5	2.51	-	-	-	-	0.89	11.1	-	34.1
$1/A_5$	BDA	-	-	91.2	-	-	-	1.04	7.8	-	-
2PMICh8/	BA	8.1	74.2	-	-	-	-	1.90	13.5	-	0.11
$1/A_6$	BDA	-	-	86.39	-	-	-	6.6	7.0	-	-

Table 37. Hydrolysis of iodoamino acid peptides with pandreatin (cont.)

Peptide	Solvent	^{131}I-activity of products of hydrolysis (%)								T_3/T_4	Ratio, R, of MIT/DIT
		MIT	DIT	I-tyrs	T_4	T_3	I-thyrs	I^-	Remainder		
PMICh8/	BA	9.4	58.4	-	-	-	8.54	5.8	17.9	-	0.16
$1/A_8$	BDA	-	-	80.3	1.73	1.27	-	4.5	13.2	0.73	-
2PMICh8/	BA	-	82.3	-	-	-	-	-	17.7	-	0
$1/A_8$	BDA	-	-	93.6	-	-	-	0.73	6.7	-	-
PMICh8/	BA	43.3	10.2	-	-	-	17.7	7.3	21.5	-	4.23
$1/N_{4-7}$	BDA	-	-	76.6	1.57	0.36	-	17.0	5.4	0.23	-
2PMICh8/	BA	56.6	26.3	-	-	-	-	3.20	13.9	-	2.15
$1/N_{5-7}$	BDA	-	-	91.9	-	-	-	4.45	3.6	-	-
PMICh8/	BA	69.8	6.2	-	-	-	6.1	3.27	14.7	-	11.26
$1/N_8$	BDA	-	-	82.8	8.75	1.00	-	2.72	4.7	0.11	-
2PMICh8/	BA	68.8	8.5	-	-	-	-	4.07	18.6	-	8.08
$1/N_8$	BDA	-	-	86.3	-	-	-	-	13.9	-	-
PMICh8/	BA	41.8	8.0	-	-	-	35.6	4.28	10.3	-	5.21
$1/N_9$	BDA	-	-	79.6	-	-	-	5.00	15.4	-	-
2PMICh8/	BA	63.2	10.1	-	-	-	11.9	3.00	11.8	-	6.26
$1/N_9$	BDA	-	-	86.3	0.7	-	-	2.70	10.3	-	-
2PMICh8/	BA	2.80	62.7	-	-	-	-	5.17	29.3	-	0.04
$1/O_{2-3}$	BDA	-	-	90.6	-	-	-	4.20	5.2	-	-
2PMICh8/	BA	54.8	18.8	-	-	-	-	2.42	24.0	-	2.92
$1/C_6$	BDA	-	-	86.1	0.42	-	-	3.34	10.1	-	-
PMICh8/	BA	13.8	4.8	-	-	-	-	-	81.4	-	2.90
$1/C_7$	BDA	-	-	17.1	0.18	-	-	6.12	82.7	-	-
2PMICh8/	BA	-	?	-	-	-	-	-	100	-	-
$1/C_7$	BDA	-	-	-	-	-	-	-	100	-	-

Table 37. Hydrolysis of iodoamino acid peptides with pancreatin (cont.)

Peptide	Solvent	^{131}I-activity of products of hydrolysis (%)								T_3/T_4	Ratio, R, of MIT/DIT
		MIT	DIT	I-tyrs	T_4	T_3	I-thyrs	I^-	Remainder		
PMICh8/	BA	53.6	3.0	-	-	-	13.2	-	30.2	-	18.2
$1/C_8$	BDA	-	-	69.7	-	-	-	0.54	29.8	-	-
2PMICh8/	BA	61.5	3.5	-	-	-	-	-	35.0	-	17.5
k/C_8	BDA	-	-	67.6	-	0.36	-	1.22	30.8	-	-
2PMICh8/	BA	19.7	43.7	-	-	-	13.4	6.88	16.3	-	0.45
$1/C_{16}$	BDA	-	-	68.0	0.58	-	-	-	31.4	-	-
2PMICh8/	BA	6.0	43.2	-	-	-	-	-	50.8	-	0.14
$1/C_{17}$	BDA	-	-	?	-	-	-	-	100	-	-
2PMICh8/	BA	64.9	5.3	-	-	-	-	3.84	26.0	-	12.19
C_{19-20}	BDA	-	-	90.1	-	-	-	1.72	8.2	-	-
2PMICh8/	BA	72.5	-	-	-	-	7.32	3.33	19.8	-	-
$1/C_{22}$	BDA	-	-	90.0	0.95	1.02	-	2.95	5.1	1.14	-

active areas could be easily identified from the histogram. Occasionally standards were added to improve the separation of the iodoamino acids (Plaskett, 1964).

After each hydrolysis the quantity of radioactivity on the origin was checked to ensure that hydrolysis was complete. Also the quantity of activity not in any peak was low in a good hydrolysis; usually less than 2-3%. The iodothyronine peak in BA was near the solvent front and contained more radioactive material than was found in the sum of the two iodothyronine peaks separated in BDA.

V.1.6. Selection of peptides: Of the 60-70 iodoamino acid peptides visible on autoradiography, some were very low in activity and some were only partially separated. Only peptides which were high in activity and which were easily isolated were selected for further investigation.

V.2. Iodoamino acid content of radioactive peptides

The difficulties encountered in the hydrolysis of thyroglobulin were also experienced in hydrolysis of the peptides. Hydrolyses were carried out for 48 hr. at 37° but some peptides remained largely unhydrolysed in this time. It was noticed that the peptides varied in their susceptibility to pancreatin. C_7 remained consistently over 80% unhydrolysed, whereas the basic peptides A_8, A_6 and A_5 were completely hydrolysed.

Unhydrolysed, or partially hydrolysed, peptide would sometimes 'mimic' free iodoamino acids. However, by analysis of the hydrolysate in the two solvent systems, and by comparison of the percentages of iodoamino acids in each case it was possible to estimate the degree of hydrolysis. Table 37 lists the main peptides examined, their susceptibilities to pancreatin and, where possible, their iodoamino

Table 38. Hydrolysis of iodoamino acid peptides with pronase

Peptide	Conditions	^{131}I-activity of products of hydrolysis (%)								Ratio, R, of MIT/DIT + MIT as percentage
		MIT	DIT	I-tyrs	T_4	T_3	I-thyrs	I^-	Remainder	
9PMIChB/	Pronase BA	94.4	0.92	-	-	-	-	1.24	3.5	99.0
A_5	18 hr. BDA	-	-	97.4	-	-	-	1.01	1.6	-
	Pronase BA	16.1	78.0	-	-	-	-	2.30	3.6	17.1
A_6	18 hr. BDA	-	-	99.0	-	-	-	0.65	0.4	-
	Pronase BA	9.0	85.6	-	-	-	-	1.88	3.5	95.1
A_8	18 hr. BDA	-	-	94.9	-	-	-	1.54	3.6	-
	Pronase BA	77.7	16.4	-	-	-	-	1.87	4.0	82.5
N_8	18 hr. BDA	-	-	95.8	-	-	-	1.59	2.6	-
	Pronase BA	73.6	15.1	-	-	-	-	1.80	9.5	83.0
N_9	18 hr. BDA	-	-	90.0	-	-	-	2.43	7.7	-
	Pronase BA	19.1	67.2	-	-	-	-	2.36	11.3	22.1
C_7	18 hr. BDA	-	-	72.3	-	-	-	1.44	26.3	-
	Pronase BA	8.2	72.8	-	-	-	-	15.4	3.6	10.1
C_7	90 hr. BDA	-	-	81.0	1.64	0.45	-	7.60	9.3	-
	Pronase BA	58.4	9.2	-	-	-	-	1.31	31.1	86.2
C_{8a}	18 hr. BDA	-	-	65.5	-	-	-	1.33	33.2	-
	Pronase BA	69.9	15.8	-	-	-	-	10.2	4.1	81.5
C_{8a}	90 hr. BDA	-	-	87.5	1.04	-	-	6.51	5.0	-
	Pronase BA	3.51	12.60	-	-	-	-	3.03	80.9	21.8
C_8	18 hr. BDA	-	-	24.7	-	-	-	1.65	73.7	-
	Pronase BA	8.90	72.1	-	-	-	-	13.17	5.8	11.0
C_8	90 hr. BDA	-	-	76.1	0.61	-	-	9.92	13.4	-
	Pronase BA	5.76	76.4	-	-	-	-	3.67	14.1	7.0
C_{17}	18 hr. BDA	-	-	71.5	-	-	-	3.39	25.1	-

Table 38. Hydrolysis of iodoamino acid peptides with pronase (cont.)

Peptide	Conditions	^{131}I-activity of products of hydrolysis (%)								Ratio, R, of MIT/DIT + MIT as percentage
		MIT	DIT	I-tyrs	T_4	T_3	I-thyrs	I^-	Remainder	
9PPMICh8/	Pronase BA	4.91	77.4	-	-	-	-	11.90	7.8	6.0
C_{17}	90 hr. BDA	-	-	71.2	1.22	0.52	-	13.11	14.0	-
	Pronase BA	82.1	8.76	-	-	-	-	1.69	7.4	90.4
C_{19}	18 hr. BDA	-	-	20.8	-	-	-	1.79	77.4	-
	Pronase BA	68.6	13.7	-	-	-	-	13.37	4.3	83.4
C_{19}	90 hr. BDA	-	-	70.5	1.45	-	-	11.53	17.5	-
	Pronase BA	81.8	5.72	-	-	-	-	1.99	13.5	93.5
C_{22}	18 hr. BDA	-	-	100.0	-	-	-	-	0	-

Table 39. Electrophoresis at pH 8.2 of iodoamino acid peptides from βPMICh8

Where the peptides separated into more than one radioactive species the activities were summed separately.

Peptide	^{131}I-activity in each peak (%)		
	1	2	3
A_5	82.1	17.9	-
A_6	74.9	25.1	-
A_8	65.0	35.0	-
N_8	67.9	22.3	8.7
N_9	64.1	35.9	-

Table 40. Demonstration that each peptide from βPMICh8 contains only one of the iodotyrosines

The peptides were hydrolysed with pronase (a) before and (b) after purification by electrophoresis at pH 8.2 and the MIT content expressed as a percentage of the total iodotyrosine content.

Peptide	MIT-content of iodotyrosines (%)	
	(a)	(b)
A_5	99.03	99.28
A_6	17.10	4.28
A_8	9.50	5.48
N_8	82.70	98.97
N_9	83.0	-
N_{9a}	-	97.15
N_{9b}	-	98.76

acid content. Some of the analyses were carried out on peptides from different preparations of ^{131}I-thyroglobulin.

Examination of the iodoamino acid contents of the peptides, even at low levels of hydrolysis, showed that one of the iodoamino acids tended to predominate except in those spots which were known to be heterogeneous.

Table 38 lists the iodoamino acid contents of peptides from later preparations which were hydrolyzed with pronase. Peptides A_5, A_6, A_8, N_8, N_9 and C_{22} were hydrolyzed for 18 hr. This was not sufficient for the remaining peptides, whose hydrolyses were continued for a total of 90 hr.

The monoiodotyrosine content of the peptides, expressed as a percentage of the total iodoamino acid released is given in Table 40, column (a). Over 80% of one or the other iodoamino acid is present in each peptide. From this arose the suggestion that each peptide, when pure, contains only one of the iodoamino acids. Proof of this finding would confirm the hypothesis that α-chymotrypsin hydrolyses the peptide bond next to each of the aromatic residues in thyroglobulin, releasing peptides containing one aromatic residue.

When peptide hydrolysis had been improved by the later use of pronase, this problem was re-examined. Samples of peptides A_5, A_6, A_8, N_8 and N_9 from preparation 11PMICh8 which had been hydrolyzed with pronase and their iodoamino acid contents found (Table 40) were dissolved in methanol:ammonia and spotted on to 2" strips of Whatman 3MM paper. These strips were wetted with 0.07M sodium barbitone buffer, pH 8.2 (Smith, 1960) and subjected to electrophoresis at 2,000 v. for 200 min. After drying at 40°, the strips were scanned for the radioactive areas.

It was found (Table 39) that the peptides, as isolated,

contained small amounts of contaminating material. Peptide N_9 was shown to comprise two peptides.

The main radioactive areas on these strips were eluted (2 from N_9) and subjected to pronase hydrolysis. Comparison of the two parts of Table 40 reveals that the contamination of the major iodotyrosine decreased from an average of 12% to no greater than 2.2%. Each peptide contains only one of the iodotyrosines as expected in products of chymotryptic digestion.

V.3. Determination of the average molecular weight of products of enzymic hydrolysis by estimation of peptide bonds hydrolyzed

Before further investigation of the iodoamino acid peptides was undertaken, it was judged necessary to determine their molecular sizes approximately. If the peptides were large, further hydrolysis with trypsin was contemplated to bring the peptide chain to a suitable length.

The progress of enzymic hydrolysis of thyroglobulin was followed on an autotitrator by recording the quantity of dilute alkali added to maintain a constant pH. From the quantity of alkali added the number of peptide bonds hydrolyzed was found after determination of the pK of the released ionized groups.

V.3.1. Apparatus: The autotitrator (Radiometer TTT/Titrator) consisted of a water-jacketed chamber of 15-20 ml. capacity maintained at 37°. The pH of the hydrolysate was monitored by a glass electrode. Alkali was added to the chamber, from a syringe of 1 ml. capacity through fine polythene tubing. The plunger of the syringe was automatically depressed to maintain a constant pH when a difference between the set pH and the measured pH was detected. The pH was maintained constant within 0.01 pH unit. The solution was stirred

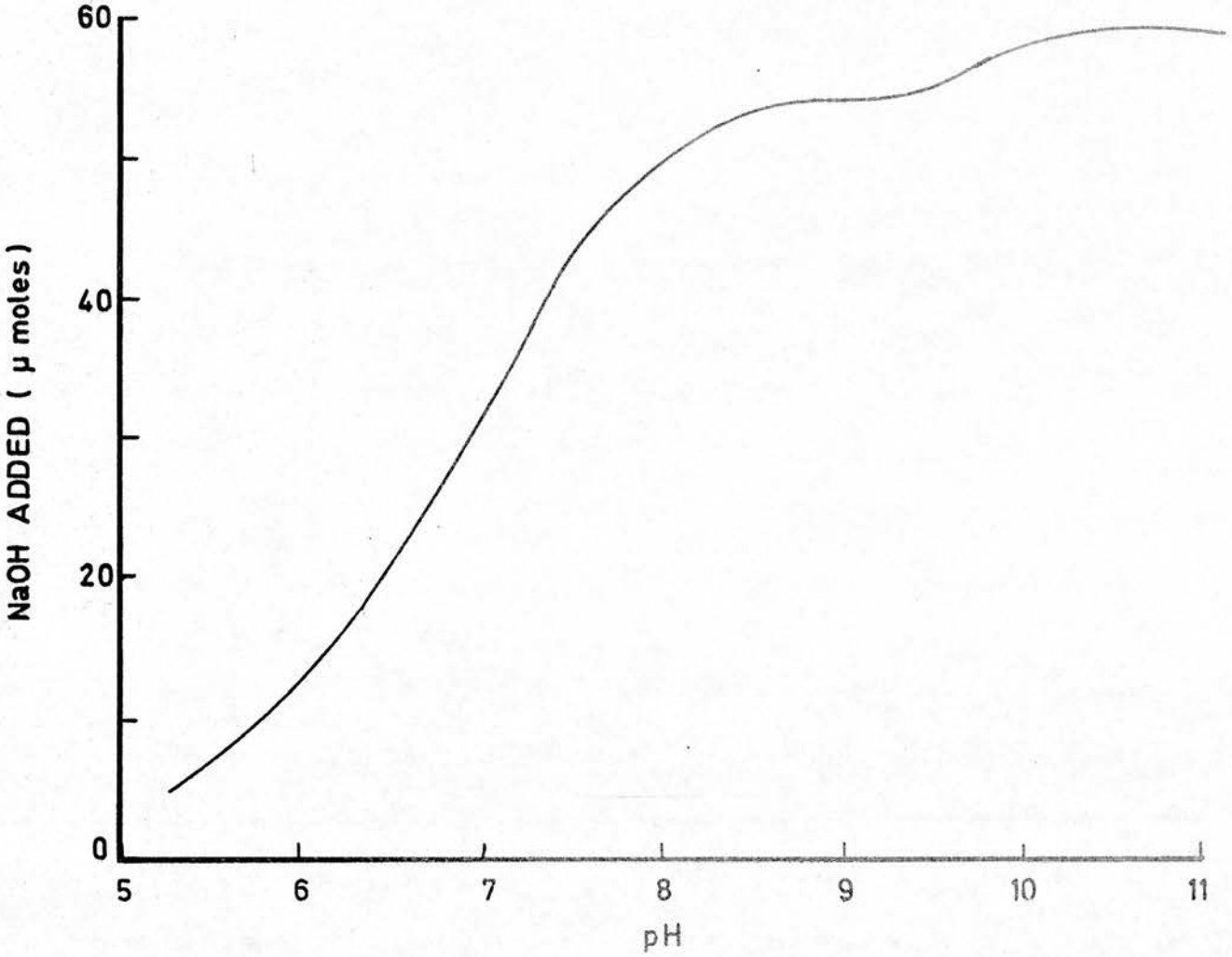


FIGURE 23 DIFFERENCE BETWEEN TITRATION CURVES OF THYROGLOBULIN BEFORE AND AFTER CHYMOTRYPSIN DIGESTION.

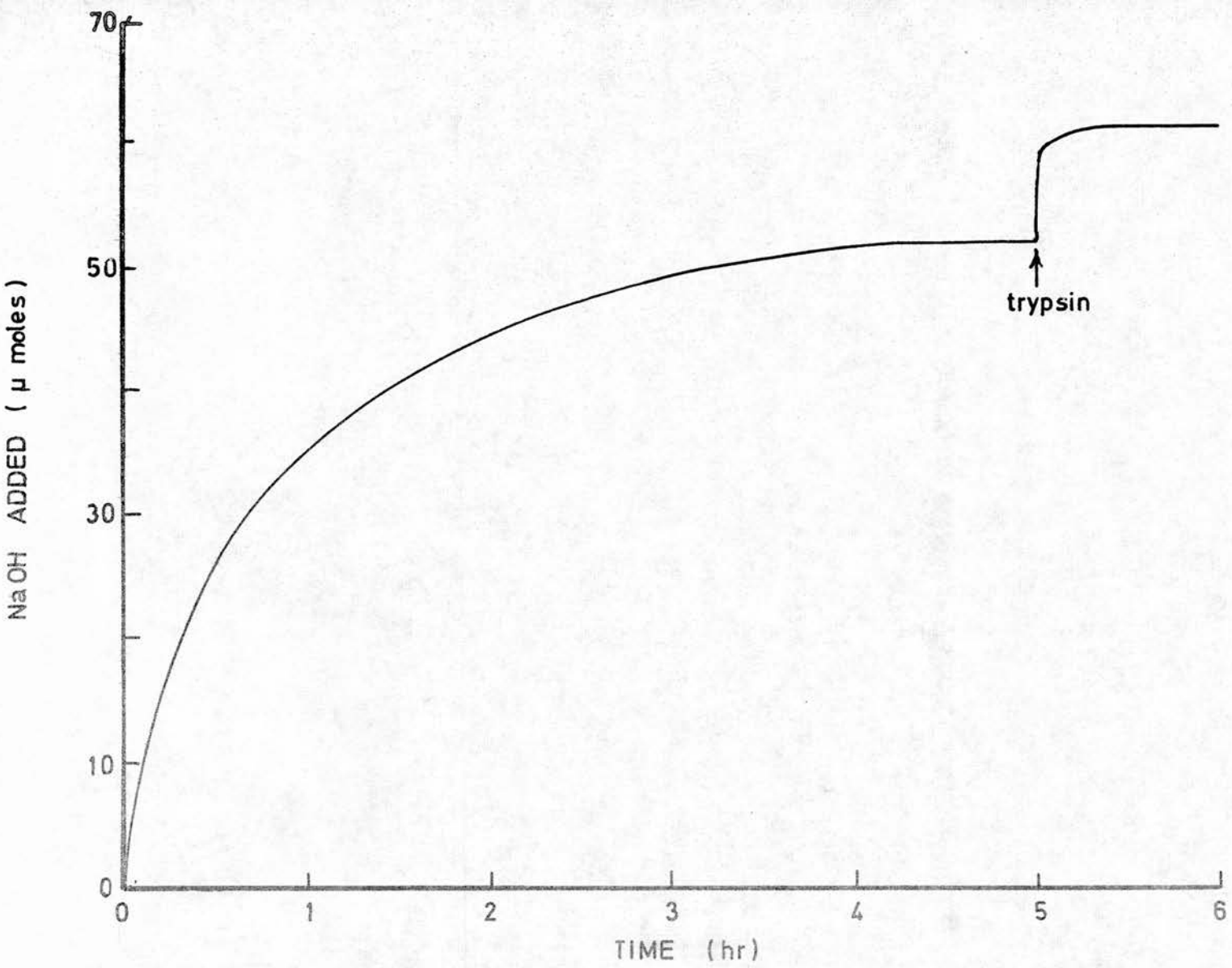


FIGURE 24 HYDROLYSIS OF SHEEP THYROGLOBULIN WITH CHYMOTRYPSIN FOLLOWED BY TRYPSIN. THE DEGREE OF HYDROLYSIS WAS DETERMINED BY THE QUANTITY OF DILUTE ALKALI ADDED AUTOMATICALLY TO MAINTAIN THE pH CONSTANT AT pH 8·6.

by bubbling nitrogen through it. The cumulative total of alkali added was shown on a chart recorder.

V.3.2. Method: Thyroglobulin (30-50 mg.) was dissolved in 10.0 ml. of 0.10N-NaCl. If the solution was to be heated to 80° for 5 min. to denature thyroidal peptidases this was done at this stage. The cooled solution was added to the chamber and the electrodes syringe and bubbler were arranged. After temperature equilibration the pH was adjusted to between 8.50 and 8.70 and the autotitrator was set for this value. α-Chymotrypsin powder (2% by weight of the thyroglobulin) was then added.

The pH was automatically kept constant and the quantity of alkali (0.1767N-NaOH) added was recorded (Fig. 24). When the addition of alkali ceased, that is when the chymotryptic hydrolysis was finished, trypsin, again 2% by weight of the thyroglobulin, was added and the further hydrolysis was followed (Fig. 24).

For titration of the initial thyroglobulin solution, or the hydrolysate, the pH was adjusted to 5.9 with dilute HCl. Alkali was added at a steady rate and the rising pH was recorded automatically.

V.3.3. Results of autotitration:

V.3.3.1. Calculation of pK of released α-amino groups: Hydrolysis proceeded by the reaction below: the exact quantity of titratable

$$-CO-NH- \xrightarrow{H_2O} -COO^- + NH_2- + H^+$$

hydrogen ion produced depended on the pH of the solution and the pK of the peptide α-amino group. The former was known and the latter was determined by titration of the thyroglobulin solution, before and after hydrolysis, from pH 5.0 to pH 11.2.

The difference in the quantity of alkali added at each half pH unit before and after hydrolysis between pH 5.0 and 11.2 was plotted against the pH (Fig. 23). This graph revealed an area of buffering

in the hydrolysate extending between pH 6.5 and pH 8 with a maximum at pH 7.3. This value was taken to be the average of the pK values of the α-amino groups released during hydrolysis.

V.3.3.2. Hydrolysis of heat-denatured thyroglobulin: 52.0 mg. of this protein required 52.9 μ moles of NaOH to maintain the pH constant at 8.61 during hydrolysis. The α-amino groups at this pH were 95.2% dissociated. In all, 55.5 μ equivalents of hydrogen ion, and, therefore, 55.5 μ moles of α-amino groups, had been released.

Thus 52.0 mg. of thyroglobulin (mol. wt. 660,000) during hydrolysis released 55.5 μ moles of α-amino groups. Each molecule of protein had 704 bonds hydrolysed.

The average molecular weight of the peptides produced by chymotryptic hydrolysis was 940 and this was decreased to 800 after trypsin hydrolysis.

Chymotryptic hydrolysis was complete in 5 hr. and tryptic hydrolysis was complete in 2 hr. (Fig. 24).

V.3.3.3. Hydrolysis of undenatured thyroglobulin: Under the same conditions of hydrolysis α-chymotrypsin split 1,170 peptide bonds and trypsin split 746 per molecule. The difference between these values and those for the heat-denatured thyroglobulin are a measure of the activity of thyroidal proteases and peptidases.

V.4. Determination of the molecular size of peptides by gel filtration

One of the simplest methods for the determination of the molecular size of proteins and peptides is by filtration through porous gel granules, the solutes appearing in decreasing order of size in the column effluent.

V.4.1. Methods: For a compound with sufficiently large molecular

weight the volume of eluant, Ve, which has to pass through the column to elute the compound is independent of the molecular weight. For smaller molecules, which can penetrate to some extent into the gel granules, the elution volume, Vi, is larger than Ve, and it can be shown (Andrews, 1964 and Carnegie, 1965) that Ve/Vi is proportional to the logarithm of the molecular weight of the smaller molecule.

Cytochrome c was chromatographed on the columns both to check that the gel particles were evenly packed, as determined by the passage of a narrow level red band down the column, but also, as the protein was totally excluded from the grade of gel used, to give the exclusion volume, Ve, for the column and the ratio Ve/Vi known in this case as $R_{cyt\ c}$.

Since each peptide was expected to terminate at one of the 400 aromatic residues in the thyroglobulin molecule (mol. wt. 660,000) the mean molecular weight of a peptide was expected at that time to be about 1,600. For this reason, Sephadex grade G-25 which excludes molecules of molecular weight greater than 10,000 and fractionates substances on the range of molecular weights from 250 to 5,000 was used.

Columns were prepared in the same way as that described on p. 66-8. The gel was swelled in 0.05M Tris (pH 7.5)/0.1M KCl and the column was washed for at least 24 hr. to allow the gel bed to settle.

The eluant was allowed to run out of the column until the top of the gel was just exposed. The peptide was added to the columns in 2 ml. buffer and allowed to run into the gel. The solute was subsequently washed on to the column with several small volumes of buffer before the eluate reservoir was fitted.

Fractions (2.0 ml.) of column eluant were collected. The fraction collected whilst the 2.0 ml. of peptide solution was

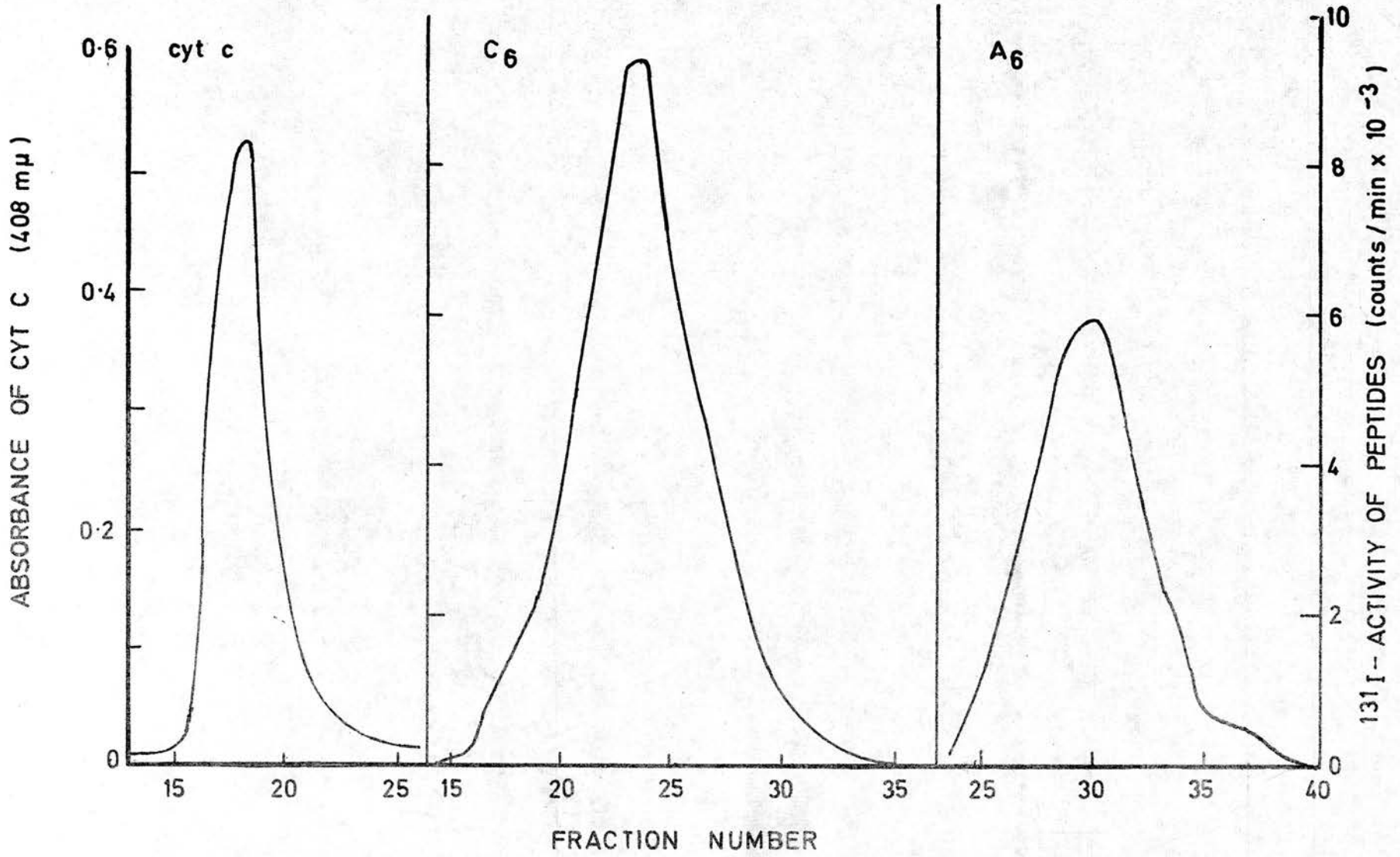


FIGURE 25 ELUTION OF CYTOCHROME C AND PEPTIDES C_6 AND A_6 FROM SEPHADEX G 25 IN PHENOL: ACETIC ACID.

entering the column was taken as fraction 1. Cytochrome c was estimated spectophotometrically at 408 mμ. Fractions of effluents containing radioactive materials were added to annular polythene cups and counted by scintillation.

The activity of each fraction was plotted against fraction number and the volume of eluant corresponding to the maximum concentration of peptide was estimated by extrapolation of the sides of the peak to an apex (Fig. 25.).

A sample of radioactive thyroglobulin was eluted from the column with a Ve of 38.0 ml. To determine the void volume, Vo, of the columns, that is, the total accessible volume in and outside the dextran beads, ammonium sulphate was added to the column and the eluate was assayed for sulphate ion by the addition of a few drops of acid 5% $BaCl_2$. The maximum concentration of ammonium sulphate occurred in fraction 40, giving a Vo for the column of 80 ml. Two peptides A_5 and C_7 were eluted from the columns. These two peptides had values of Vi of 110 ml. and 72.4 ml. respectively. They had partially adsorbed to the dextran and had eluted with an artificially high Vi. This type of adsorption is found particularly with small peptides which contain aromatic residues (Carnegie, 1965).

The adsorption was avoided by eluting the peptides from the column in the phenol:acetic acid:water (2:8:10, w:v:v) solvent introduced by Synge and Youngson (1961). This acid solvent decreases the ionisation of the phenolic hydroxyl group and makes electrostatic attraction to the dextran less likely. It is also probable that the phenol acts as a 'competitor' for the attractive sites on the dextran and by virtue of its high concentration virtually replaces the peptides. In this solvent the peptides A_5 and C_7 eluted in volumes less than the void volume.

Table 41. Peptides used by Carnegie (1965) to calibrate Sephadex G-25 columns for molecular weight determinations

Number: corresponding to those in Fig. 26	Amino acid, peptide or protein	Mol. wt.	$R_{cyt\ C}$ *
1	Cytochrome C	12,400	1.00
2	Insulin	5,800	0.995
3	Glucagon	3,500	0.950
4	α-Melanocyte stimulating hormone	1,681	0.915
5	Bacitracin	1,411	0.84
6	β-Asp^{1}-Val^{5}-Angiotensin II	1,031	0.805
7	Gly^{6}-Bradykinin	1,029	0.79
8	Val.Tyr.Val.His.Pro.Phe	760	0.74
9	His.Phe.Arg.Trp.Gly	701	0.73
10	Gly.Trp	261	0.535
11	β-Ala	89	0.52

* $R_{cyt\ C}$ is the ratio of the elution volume of cytochrome C (on G-25 equal to the exclusion volume for the column, Ve) to the elution volume of the other standards which are not fully excluded, Vi.

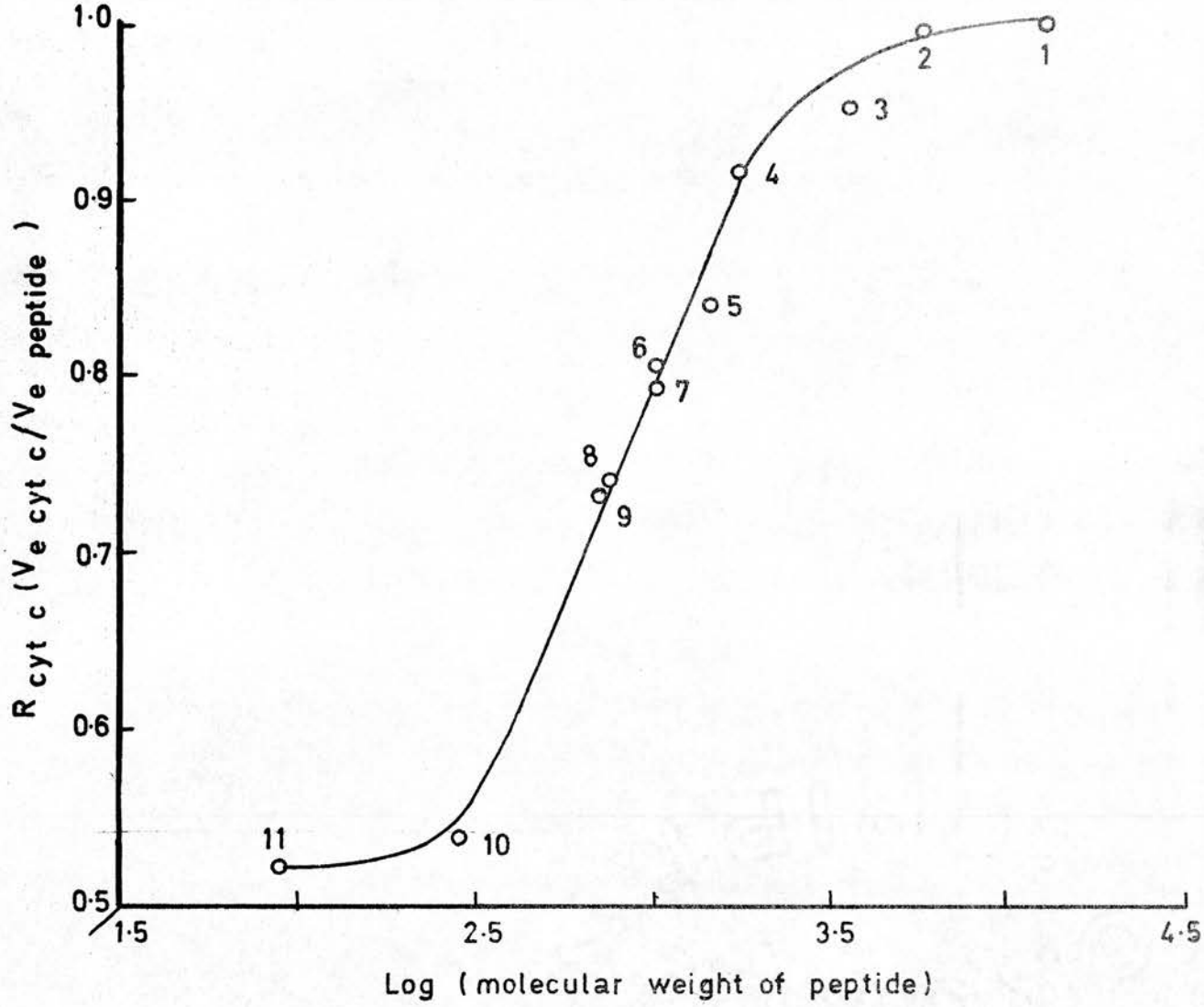


FIGURE 26 STANDARD CURVE FOR THE ESTIMATION OF PEPTIDE MOLECULAR WEIGHTS FROM THEIR $R_{CYT\ C}$ VALUES. DRAWN FROM DATA OF CARNEGIE (1965).

Table 42. Molecular weights of iodoamino acid peptides from 3PMICh8 found by gel filtration on Sephadex G-25 after comparison with a standard curve (Fig. 26)

Peptide	Molecular Weight
A_5	< 300
A_6	360
A_8	490
A_8T_2 *	< 300
C_6	800
C_7	776
C_{17}	676

* After trypsinization, Section V.6

Table 43. Distribution of molecular size in an α-chymotrypsin hydrolysate of sheep thyroglobulin separated on Sephadex G-25

Data from Fig. 27

Range of molecular weight	^{131}I-activity (% of total)
> 10,000	5.9
2,000-10,000	4.6
1,500-2,000	2.9
1,000-1,500	8.4
500-1,000	24.9
250-500	27.4
< 250	26.0

However, when the columns were thoroughly washed in this solvent the Ve was found to have decreased because dextran gels swelled to a lesser extent in less aqueous solvents. Ve for thyroglobulin was now 34.8 ml. and for cytochrome c 35.4 ml. (Fig. 25). The values of Vi for samples of A_5 and C_7 which were eluted from the column in the organic solvent, were 58.0 ml. and 46.7 ml. The void volume of the column was 92 ml. as determined by elution of $Na^{131}I$. Several other peptides were chromatographed in the same way (Fig. 25).

V.4.2. Results: The molecular weights of these peptides were estimated from a graph of data extracted from a paper by Carnegie (1965). Using Sephadex G-25 and a phenol:acetic acid:water eluant, Carnegie had chromatographed several peptides of known molecular weights (Table 41). A plot of their $R_{cyt\ c}$ against log(molecular weight) showed that most of the points lay on a straight line (Fig. 26). The molecular weights corresponding to the values of $R_{cyt\ c}$ found for the iodoamino acid peptides were read off from the graph. These are listed in Table 42.

Carnegie (1965) claims that the molecular weight of each peptide can be found within an error of $\pm$10%. However, the columns used here had no standard peptides chromatographed on them. Also the separation on the columns is by differences in molecular size rather than molecular weights. In most cases these correspond closely but the iodine atoms in the iodotyrosyl residues may affect the geometry of the peptides sufficiently to influence the molecular sizes.

Nevertheless, the molecular weights obtained are sufficiently accurate to emphasize that the peptides which have been isolated and examined are of small molecular weight, in all cases, less than

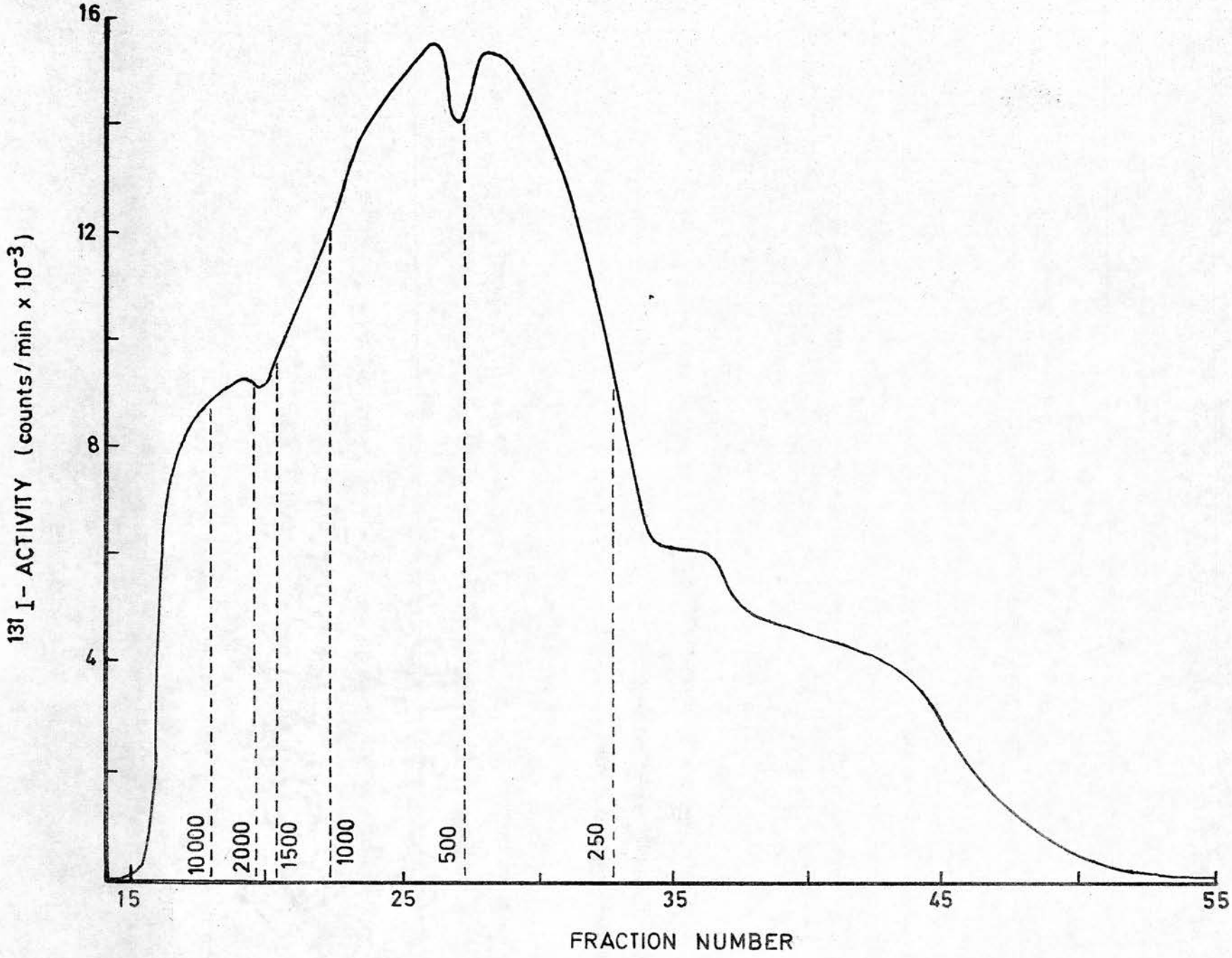


FIGURE 27 RANGE IN MOLECULAR SIZE OF SHEEP THYROGLOBULIN CHYMOTRYPTIC PEPTIDES ON SEPHADEX G 25

1,000.

This value was lower than the average expected, but the peptides isolated were not a random sample. They were, in general, those which were discrete and, in the main, low in contamination by other peptides. These were usually the peptides with highest electrophoretic mobilities and R_f values on chromatography.

V.5. Distribution of molecular size of peptides in α-chymotryptic hydrolysis

A sample of the α-chymotryptic hydrolysis from which the above peptides had been isolated (3PMIChB) was chromatographed on a G-25 column in the phenol:acetic acid solvent. The radioactivity in the eluate was spread over all the fractions from the exclusion volume to beyond the void volume. The activity in each fraction was plotted against the molecular weight for material with that elution volume (Fig. 27).

The quantity of peptide material in various ranges of molecular weight was calculated (Table 43).

It was evident that the thyroglobulin molecule had been largely hydrolysed. Most radioactive material has molecular weights in the range 300-1,000. The average molecular weight of peptides less than 1,000 is 560. This agrees with the few determinations of molecular weights of individual peptides (Table 42).

By reference to the molecular weights of mono- and diiodotyrosine, 308 and 435 respectively, it is clear that the molecular weights assigned to some of the individual peptides (Table 42) and lower molecular weight peptide material (fractions 30-50, Fig. 27) cannot be correct. The spuriously low molecular weights were not produced by contamination with free iodotyrosines because the

chymotrypsin hydrolysate contains a negligible quantity of these and the individual peptides were selected from areas of the peptide maps considerable distances from the positions occupied by the iodotyrosines.

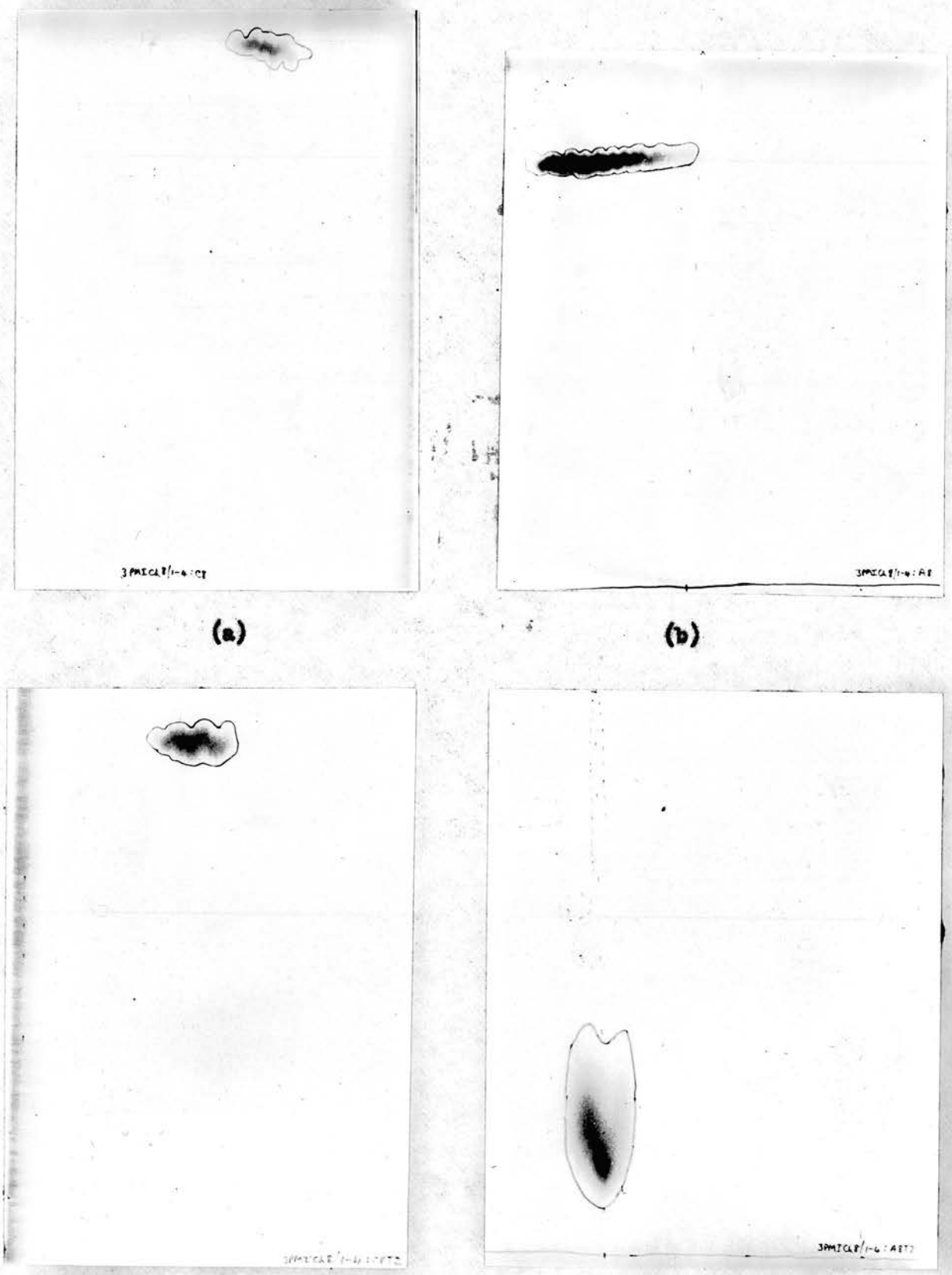
Even in a phenol:acetic acid eluant the Sephadex columns may have a residual attraction for the short iodoamino acid peptides which would lead to spuriously low molecular weights. The effect of the iodine atoms in the peptides is not known: although these contribute largely to the molecular weight they may have less influence on the molecular size, which is the criterion for separation by gel filtration.

The main conclusion of the peptide gel filtration still stands, namely, thyroglobulin is very largely hydrolysed by α-chymotrypsin and the most intensely labelled peptides are in fact of small molecular weight. Calibration of Sephadex G-25 columns under the above conditions with synthetic iodotyrosyl peptides would allow determination of the actual molecular weights of the chymotryptic peptides.

V.6. Trypsinization of peptides

Both chymotrypsin and trypsin were considered for the initial hydrolysis of thyroglobulin, but the former was preferred because each peptide was expected to contain only one iodoamino acid. However, as there are 430 sites for chymotrypsin action and 1,066 for trypsin, the latter would release more peptides, each of a smaller size. Investigation and further analysis of the smaller peptides would be much easier. For this reason, the action of trypsin on three peptides was studied.

A basic, a neutral and an acidic peptide were isolated from



(a) (b)

(c) (d)

Plate 10. Autoradiograms, after peptide mapping, of peptides C_8 and A_8 before trypsinization, (a) and (b), and after exposure to trypsin, (c) and (d), respectively

3PMICh8/1-4. These were A_8, N_8 and C_8 respectively, containing 1.31%, 2.44% and 1.28% of the activity on the peptide map and almost completely hydrolysed by pancreatin. A_8 contained diiodotyrosine, the other two monoiodotyrosine. All three spots on the peptide map were small and discrete, and the peptides were not subjected to further purification.

V.6.1. Methods: A sample of each peptide was peptide mapped and autoradiographed.

Similar samples of the peptides were taken up in 200 µl. of the Tris buffer used for chymotryptic hydrolysis (Section IV.1.2. p. 90). Trypsin (Sigma) (0.2 mg.) was added to each and the hydrolysis continued at 37° for 2 hr. The autotitration data had indicated that trypsin hydrolysis was complete within 2 hr. The hydrolysate was peptide mapped (for 2 hr. to stop free amino acids running off the paper) and autoradiographed.

V.6.2. Results: N_8 and C_8 (Plate 10) appeared to be unchanged after trypsin hydrolysis. Although it is possible that trypsin removes a peptide containing both a basic and acidic amino acid, thus leaving the remaining iodoamino acid peptide with the same charge, it is unlikely that the remaining peptide would have the same R_f value in BA.

A_8 was further hydrolysed by trypsin, the active material becoming electrically neutral with a high R_f in BA (Plate 10). Spraying the peptide map with ninhydrin revealed a faint spot in position indicated for a basic amino acid.

A sample of the trypsinized A_8 was chromatographed on G-25 in the phenol:acetic acid solvent and its elution volume indicated a drop in apparent molecular weight from 490 to less than 300.

Thyroglobulin contains 440 basic amino acids and if the

chymotryptic peptides were large, each should contain one or more basic residues. Of the three peptides selected, only one, a very basic one, contained a basic amino acid. This provided indirect evidence for the size of the chymotryptic peptides. As these are, in fact, quite small no further hydrolysis was attempted before investigation.

V.7. In vitro iodination of peptides by sheep thyroid slices

Each peptide, as isolated, contains one iodoamino acid (but see pp. 153-4) and represents one of the sites on the thyroglobulin peptide chain where iodination takes place and where the further coupling of iodotyrosyls to iodothyronines will, in some cases, occur.

Both by the extent of darkening on autoradiograms, and by the counting of individual peptides, it had been realised that the peptides varied considerably in their concentration of label. The reasons for this were not immediately clear, because, if each 'site' were iodinated with equal facility, the peptides should divide into two groups - one having twice the specific activity of the other, and containing diiodinated tyrosyl residue. If, however, the tyrosyl residues were not equally susceptible to iodination, their rates of iodination would be different and these would allow identification of the more active residues which would, a priori, be considered the mostlikely sites for thyroxine synthesis.

The time-course of iodination of individual peptides in thyroglobulin with tissue slices in vitro where this process might be expected to be slower than in vivo, was expected to reveal early increases in monoiodotyrosine levels, followed by conversion of this to diiodotyrosine. Under the conditions of peptide mapping, the two iodinated forms of a peptide will separate (Table 46) and, if both forms are present, will exist

as a 'pair'. Where such a pair was suspected a decrease in the initially high activity of the monoiodotyrosine-containing 'partner' would provide good circumstantial evidence for their relationship.

Sheep thyroid slices were incubated with $Na^{131}I$ for different lengths of time and the uptake of the iodide, the levels of iodo-amino acids and the activities of selected peptides in the isolated thyroglobulin were determined. Data from previous preparations, with incubation times of 8 and 24 hr. are included for comparison.

V.7.1. Methods:

V.7.1.1. Iodination conditions: Portions (250 mg.) of sheep thyroid slices were added to 2.0 ml. of KRB in tubes which were gassed and kept in ice.

One sample was warmed to 37° in a water bath and 200 µl. of a solution of $^{131}I^-$, containing 154 µC added. After 1 min. the tube was cooled rapidly and the contents were transferred with washing to a cooled all-glass homogenizer. The homogenate was made up to 5.0 ml. of which 0.5 ml. was retained for determination of iodide uptake. The remainder was centrifuged at 125,000 g for 40 min. at 0°. The clear supernatant was transferred to 1 cm. diameter dialysis tubing.

The other samples had the same quantity of iodide added, and after regassing, were incubated at 37° for 20 min., 60 min. or 280 min. After incubation, the samples underwent the same treatment as the first sample.

The soluble proteins were exhaustively dialysed against 0.1M ammonia and freeze-dried.

V.7.1.2. Determination of iodide uptake: Samples (0.5 ml.) of the homogenates were added to 0.5 ml. of a 1% solution of pronase (Section III.8.1., p. 87). After 18 hr. at 37° samples of the

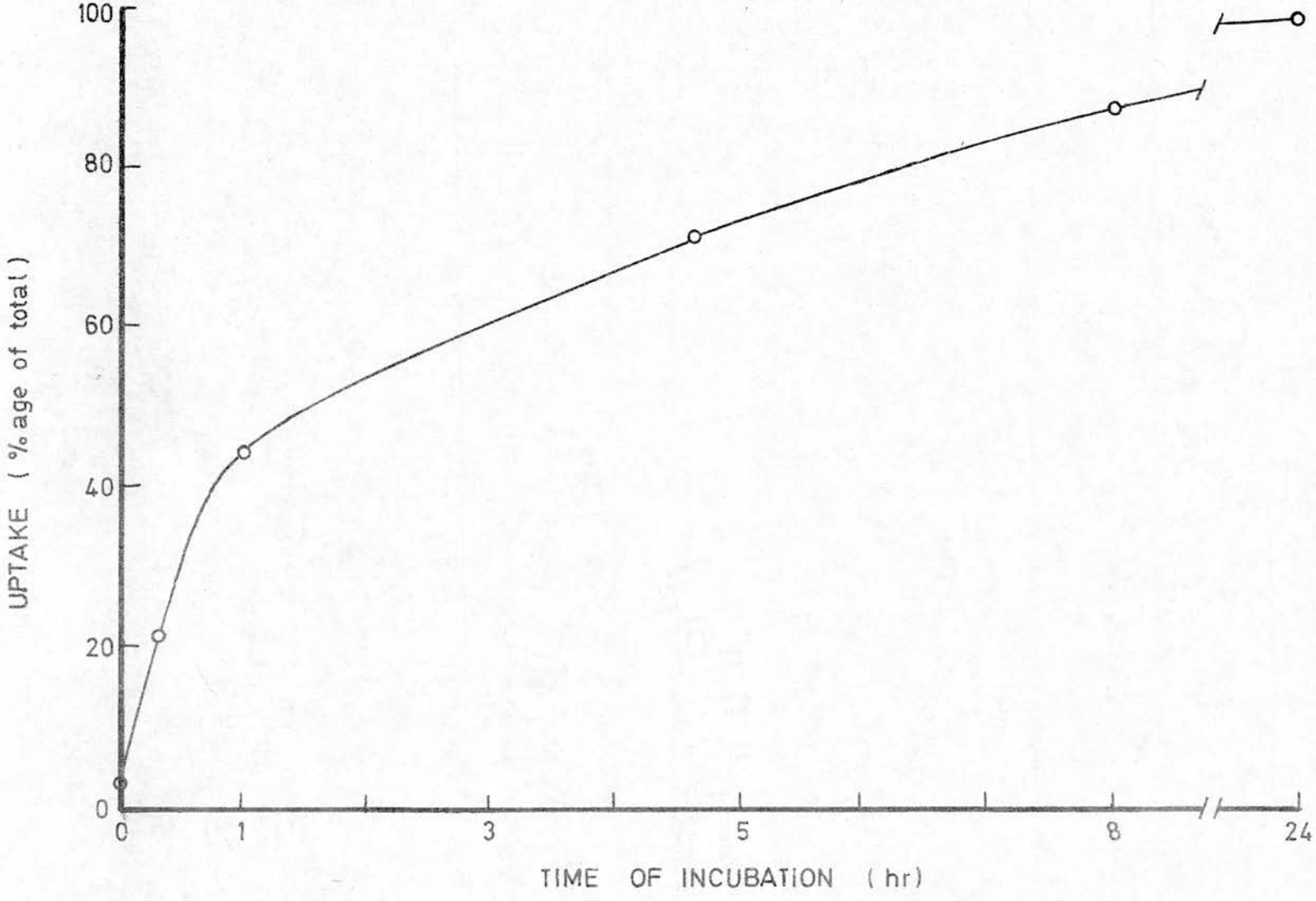


FIGURE 28 ORGANIFICATION OF IODIDE BY SHEEP THYROID SLICES.

hydrolysate were chromatographed in BA and BDA. Counting of the dried chromatograms allowed determination of the percentage of iodide taken up into organic linkage.

V.7.1.3.: Iodoamino acid content of isolated thyroglobulin: Small samples of the freeze-dried protein were dissolved in 0.5 ml. of an 0.5% solution of pronase. A 6 hr. hydrolysis was followed by chromatography of the products in BA and BDA. After counting, the percentage activity in each iodoamino acid was calculated.

V.7.1.4. Estimation of the radioactivity in each peptide: Chymotryptic hydrolysis, peptide mapping, autoradiography and cutting out of the peptide areas was identical to the details in Sections IV.1.2. p. 90, IV.2.2. p. 92, IV.2.5. p. 94, and V.1.1. p. 104, respectively. As the peptides chosen were of high activity, it was usually possible to count each to less than 3% error.

Six peptide maps were produced from each sample of thyroglobulin. Nineteen peptides were cut out and counted from each of these peptide maps. The percentage of the activity in each peptide with its standard deviation was calculated. The activity of the peptides from thyroglobulin which had undergone iodination for 1 min. only was too low to allow counting of the individual peptides.

V.7.2. Results:

V.7.2.1. Uptake of iodide into organic binding in slices: The initial uptake of iodide was rapid and approximately linear over the first hour, approaching 75% after 5 hr. incubation (Fig. 28). Comparable figures of uptake for incubations of 8 and 24 hr. are included. The latter figures are the activities of the iodine not removed during dialysis of the soluble proteins released by homogenization of thyroid slices. By 24 hr. virtually all the free iodide had been organified by the slices.

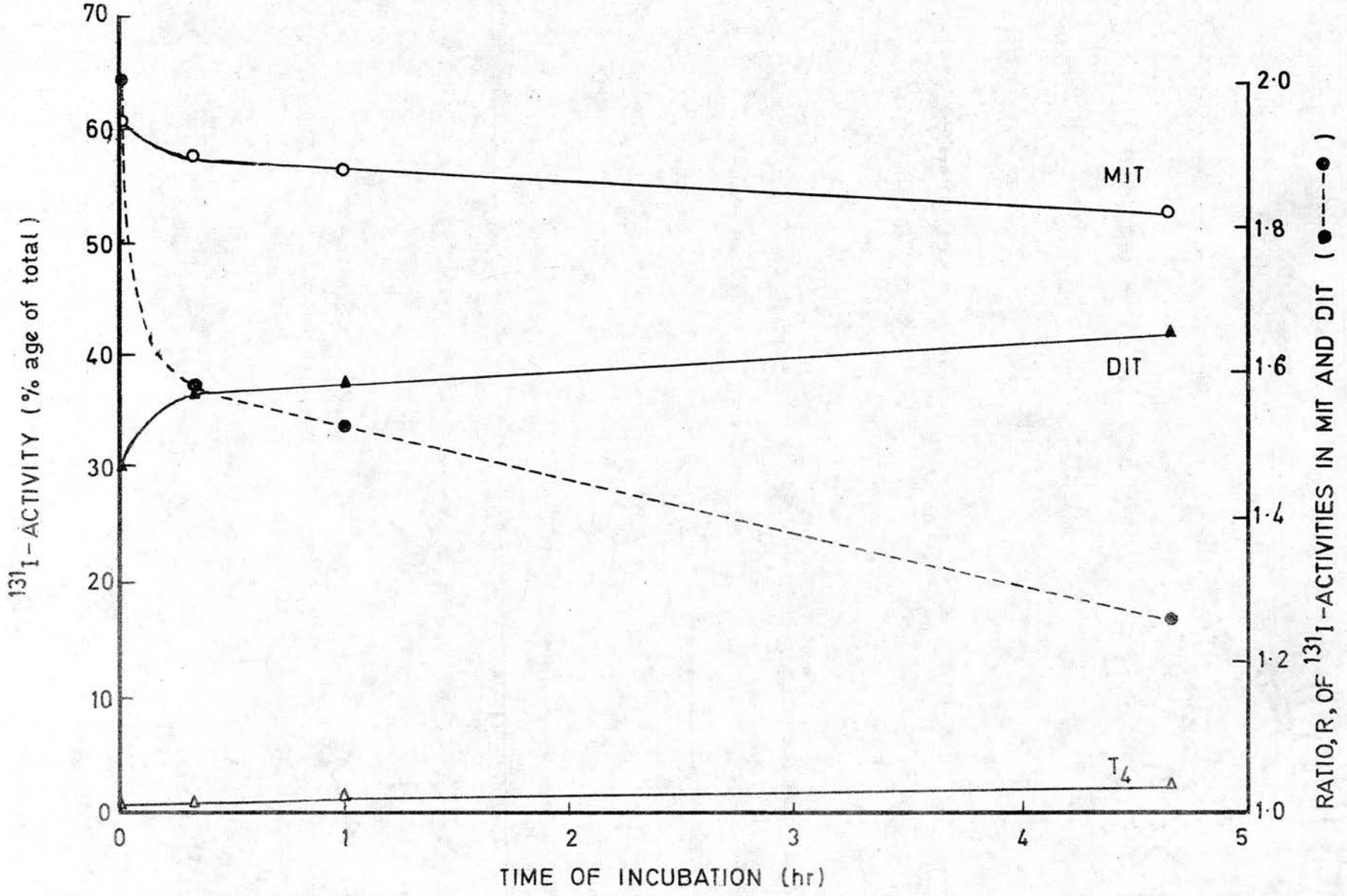


FIGURE 29 IODOAMINO ACID CONTENT OF THYROGLOBULIN FROM SHEEP THYROID SLICES.

Table 44. Effect of time on the in vitro iodination of iodotyrosyl peptides

Peptide	Major iodotyrosine	Percentage ^{131}I-activity in peptide ± standard deviation					
		Time of incubation of slices (hr.)					
		0.33	1.00	4.67	8.0	24(i)	24(ii)
A_1		1.68 ±0.67	0.326±0.069	0.05	0.31 ±0.19	-	0.59 ±0.156
A_5	MIT	2.34 ±0.159	2.582±0.237	2.465±0.287	1.60 ±0.476	1.382±0.070	1.27 ±0.42
A_6	DIT	3.19 ±0.189	2.868±0.531	3.751±0.350	2.523±0.864	6.723±0.415	4.18 ±2.54
A_8	DIT	2.35 ±0.159	2.383±0.218	2.41 ±0.193	1.893±0.438	5.10 ±0.429	4.29 ±0.635
N_6	MIT	2.33 ±0.329	2.140±0.191	1.378±0.315	3.465±1.506	1.706±0.134	1.97 ±0.587
N_7	MIT	1.833±0.248	1.670±0.188	1.686±0.391			
N_{8a}	MIT	1.22 ±0.273	0.833±0.293	0.497±0.153	-	0.877±0.136	-
N_8	MIT	3.99 ±0.098	4.31 ±0.394	4.695±0.255	3.76 ±1.283	1.852±0.097	3.128±1.074
N_{9a}		1.69 ±0.466	-	-	-	-	-
N_9	MIT	2.88 ±0.307	3.700±0.511	3.117±0.357	3.547±1.197	3.260±0.365	5.60 ±2.84

Table 44. Effect of time on the in vitro iodination of iodotyrosyl peptides (cont.)

Peptide	Major iodotyrosyl	Percentage ^{131}I-activity in peptide ± standard deviation					
		Time of incubation of slices (hr.)					
		0.33	1.00	4.67	8.0	24(i)	24(ii)
C_{6a}		2.12 ±0.693	2.928±0.301	2.265±0.242	-	-	-
C_6		3.776±0.740	2.518±0.224	2.467±0.228	3.203±0.955	-	-
C_7	DIT	4.943±0.924	4.435±0.325	3.22 ±0.191	3.747±2.552	2.57 ±0.275	2.59
C_8	DIT	4.910±1.016	6.06 ±0.376	1.141±0.107	1.88 ±1.655	0.901±0.107	0.89
C_9		1.76 ±0.231	1.297±0.375	4.941±0.192	-	-	-
C_{16}	DIT	2.493±0.210	2.020±0.377	1.94 ±0.206	1.99 ±1.259	0.638±0.098	0.62
C_{17}	DIT	4.871±0.426	4.10 ±0.326	4.551±0.178	1.69 ±0.698	1.278±0.100	1.26
C_{19}	MIT	2.150±0.686	1.958±0.244	-	-	-	2.01
C_{20}		1.697±0.309	2.567±0.199	2.841±0.379	2.69 ±1.556	-	-
C_{22}	MIT	1.161±0.307	1.295±0.260	1.016±0.237	-	-	0.85

V.7.2.2. Incorporation of $^{131}I_2$ into iodotyrosyl residues in thyroglobulin: The quantities of ^{131}iodine in monoiodotyrosine, diiodotyrosine and thyroxine released by pronase hydrolysis were calculated as percentages of the total ^{131}iodine present in the purified samples of thyroglobulin.

The uptake of ^{131}iodide in the first minute into monoiodotyrosine was twice as rapid as the uptake into diiodotyrosine. After longer time intervals, although the absolute quantities of ^{131}iodide organified continued to increase, the percentage activity in monoiodotyrosine decreased with a concomitant rise in the percentage activity of diiodotyrosine (Fig. 29). This difference is best shown in the ratio, R, of activities of monoiodotyrosine to diiodotyrosine, which fell from 2.03 at 1 min. to 1.23 after 280 min. Earlier preparations had revealed that this ratio dropped to 0.90 after 8 hr. and may reach the in vivo ratio of 0.75 (Lissitsky, 1966) after 24 hr.

The radioactivity of the thyroxine rose linearly to 2% after 280 min. Pronase hydrolysis of thyroglobulin iodinated for 8 hr. released almost 4% of the activity as thyroxine.

Thus initially there were 4 molecules of monoiodotyrosine iodinated with ^{131}I for each molecule of diiodotyrosine. After 280 min. this had decreased to 2.5 molecules of monoiodotyrosine iodinated for each of diiodotyrosine. This considerable change was brought about by alterations in only a few peptides which, it was thought, could easily be identified.

V.7.2.3. Activities of the individual peptides: Only 4 peptides (Table 44) experienced a large change in their percentage activity over 4 hr. 40 min. The remainder of the peptides altered little, or not at all, in their percentage contribution to the total activity of the ^{131}I thyroglobulin.

The ratio of the ^{131}I-activities of monoiodotyrosine and diiodotyrosine fell rapidly at early times and decreased linearly from 20 min. to 5 hr. The change in ratio was considerable and such an effect was likely to be produced by an increased conversion of monoiodotyrosine to diiodotyrosine after longer times of iodination. However, this change was not identified with any particular peptide. It appears that either this effect is spread between all the peptides and cannot for this reason be detected, or that the less active peptides which have not been studied but would, of course, contribute to the total iodine in thyroglobulin are undergoing a gradual conversion from monoiodotyrosine to diiodotyrosine.

Included in Table 44 are the percentage activities of peptides from thyroglobulin labelled <u>in vitro</u> for 8 or 24 hr. Some of these values differ from those at earlier times but as the former peptides were isolated from a different preparation of sliced sheep thyroid this probably accounts for the altered values.

From the single preparation of sheep thyroid slices it appears that each peptide makes up a characteristic percentage of the total activity of each iodinated thyroglobulin molecule. This characteristic level of iodination, which is rapidly reached in less than 20 min. and which does not change over 5 hr., is true equally for the mono- or diiodinated residues. Because of this it has proved impossible to show if any of the monoiodotyrosine peptides were further iodinated to identifiable diiodotyrosine peptides.

Chapter VI

IODINATION OF PEPTIDES

As the question of whether monoiodotyrosine peptides were converted to diiodotyrosine peptides *in vivo* had not yet been settled, it was decided to tackle this by a direct method. Chemical iodination of the monoiodotyrosine peptides was to be followed by attempts to determine if the products were identical with diiodotyrosine peptides which had been previously isolated.

It was assumed that the major effect on the electrophoretic mobility of an amino acid or peptide of the addition of a single iodine atom would be due to the relative increase in the ionisation constant of the phenolic hydroxyl. Using N-acetyl-L-tyrosine (NAT) as a model compound it was hoped, if the above assumption proved to be valid, that changes in mobility (and chromatographic R_f values) after iodination could be predicted thus allowing identification of the iodinated products of monoiodinated peptides.

As a corollary to this work, iodination of a monoiodotyrosine peptide (or the monoiodotyrosine from such a peptide) with $^{131}I_2$ was expected to allow the quantity of such a peptide to be determined. This in turn would show whether all of such a peptide was present in the one form.

VI.1. Methods:

VI.1.1. Production of $^{131}I_2$ by exchange of $^{127}I_2$ with $^{131}I^-$: Iodination of the very small quantities of peptide isolated from peptide

maps required small amounts of iodine which, in turn, necessitated that its specific activity be very high. Iodide-iodine exchanges in a single aqueous phase suffered an inevitable decrease in specific activity from the dilution of the $^{131}I^-$ by the potassium iodide required to dissolve the $^{127}I_2$.

Experiments were therefore carried out in which $^{127}I_2$ in ether exchanged with carrier-free $^{131}I^-$ in the aqueous phase. $^{127}I_2$ (0.5 mg.) was added in 1.0 ml. of ether to 0.54 ml. of an aqueous solution of $^{131}I^-$ containing 250 μC. After 1 hr. the iodine was extracted five times with 0.5 ml. of ether, removing over 90% of the radioactivity and yielding a solution of $^{131}I_2$ of nearly 500 μC/mg.

This specific activity was sufficient to iodinate very small quantities of material and to allow easy detection of products.

VI.1.2. Iodination of tyrosine compounds: The material to be iodinated was dissolved in 0.5 ml. of concentrated ammonia. To this was added the $^{131}I_2$ in 1.0 ml. of ether. The mixture, in a stoppered tube, was occasionally shaken over a period of 1 hr. During this time the iodine solution became colourless; the excess iodine being converted to iodide.

The products were rotary evaporated at less than 40° and stored at -20°. Prior to paper chromatography in butanol:acetic acid (see p. 106) or electrophoresis (Section VI.6.) the products were taken up in MeOH/conc. NH_3.

VI.1.3. Detection of N-acetyl-L-tyrosine and iodinated products: N-acetyl-L-tyrosine was made up in methanol:concentrated ammonia (1:1) to different concentrations so that 40 μl. spotted on to 3MM Whatman paper, contained 1,200 μg., 128 μg., 12.8 μg., 1.3 μg. and 0.13 μg.

After drying, the spots were sprayed with α-nitroso-β-naphthol (0.1% in acetone, w:v), air-dried and sprayed with 2.2M nitric acid in

Table 45. Chemical iodination of monoiodotyrosine with iodine-131

The MIT was dissolved in 100 µl. of 25% concentrated ammonia and the iodine-131 added in 200 µl. ether. After 2 hr. the products were taken to dryness, dissolved in 50 µl. of ethanol : concentrated ammonia and chromatographed in BA

MIT (µmole)	1.33	1.33	1.33	1.33	1.33
$^{131}I_2$ (µmole)	0.67	1.33	2.66	5.33	13.3
Activity of MIT after iodination (counts/min. x 10^{-3})	92.0	133	25.1	4.4	-
Activity of DIT after iodination (counts/min. x 10^{-3})	86.3	245	547	526	33.0
Activity of iodide (counts/min. x 10^{-3})	74	177	430	1,330	2,350
Total activity of MIT, DIT and I^- (counts/min. x 10^{-3})	252	555	1,002	1,869	2,383
Total activity on chromatogram (counts/min. x 10^{-3})	257	579	1,063	2,200	3,094

acetone. Although Acher and Crocker (1952) recommend heating at 100° for 3-4 min. to identify monoiodotyrosine and diiodotyrosine, the colour with N-acetyl-L-tyrosine developed in the cold.

Only the spots containing 1,200 µg., 128 µg., and 12.8 µg. were unequivocally visible. The minimum quantity of N-acetyl-L-tyrosine, or its derivatives added to chromatograms in later experiments was 100 µg. (0.5 µmoles). This is in agreement with the recommendation of Acher and Crocker (1952).

IV.2. Determination of iodination conditions

Monoiodotyrosine (1.00 µmoles) was iodinated with 0.5 to 10 µmoles of $^{131}I_2$ per mole.

The products were chromatographed in BA, counted, visualized with $PdCl_2$ or ninhydrin/$CuSO_4$ spray and the absorbance of the products found using a Chromoscan.

With increase in the ratio of iodine to monoiodotyrosine there was increase in the amount of diiodotyrosine produced, until at a ratio between 2 and 4 moles of iodine per mole of monoiodotyrosine, all monoiodotyrosine was diiodinated (Table 45). This result was found both by counting the area where monoiodotyrosine was expected and by the colorimetric estimation of the iodoamino acids. The specific activity of the iodine atoms in monoiodotyrosine and diiodotyrosine increased to the same extent and apparently reached a peak at 4 moles $^{131}I_2$ to 1 mole monoiodotyrosine. At this ratio and greater $^{131}I_2$ to monoiodotyrosine ratios, the total quantity of ninhydrin positive material decreased. This suggests oxidation of monoiodotyrosine by the reduction of the excess iodine to iodide in the presence of the concentrated ammonia. Under the same conditions the exchange of the iodine in diiodotyrosine

with free $^{131}I_2$ was complete.

The conversion of acetyltyrosine to diiodoacetyltyrosine requires larger molar ratios of iodine than the conversion of tyrosine to monoiodotyrosine.

Samples (2 μmoles) of acetyltyrosine in 200 μl. 25% aqueous ammonia were allowed to react with 1, 2 and 4 μmoles of $^{131}I_2$ in 400 μl. of ether.

Separation of the products by electrophoresis showed the presence of both the mono- and diiodinated forms. (see below)

VI.3. Electrophoresis of tyrosine and iodotyrosines

Examination of the electrophoretic mobilities of tyrosine and N-acetyl-L-tyrosine at two pH values and after iodination was expected to demonstrate the probable way in which the mobilities of the monoiodotyrosyl peptides would change under similar conditions. Information of this type would provide useful confirmation of the results from iodination of peptides.

VI.3.1. At pH 6.5 in pyridine:acetic acid:water: Samples (5 μmoles) of tyrosine, monoiodotyrosine and diiodotyrosine were subjected to electrophoresis separately and as a mixture at pH 6.5 for 3½ hr. at 2,000 v. and 2mAmp/2.5 cm. width of paper. The tyrosines were visualized by ninhydrin.

Tyrosine and monoiodotyrosine separately and together moved 4.4 cm. to the cathode, and diiodotyrosine moved 1.9 cm. from the origin to the anode.

The electrophoretic migration of an ion is the algebraic sum of the distance moved from the origin and the movement due to electroendosmosis. As diiodotyrosine moved 1.9 cm. to the anode from the origin, and tyrosine, which has virtually zero net charge,

Table 46. Dissociation of the phenolic hydroxyl group of tyrosine, N-acetyl-tyrosine and their iodinated products at pH 6.5 and 8.2 with the corresponding mobilities during electrophoresis

Dissociation of the hydroxyl groups of the N-acetyl-tyrosines calculated from the pK values of Mayberry, Rall and Berman (1965)

		Migration rate (cm^2/KV-hr.)		Dissociation of hydroxyl (%)	
	pK	pH 6.5	pH 8.2	pH 6.5	pH 8.2
Tyrosine	10.13	0	0	0.019	0.048
Monoiodotyrosine	8.20	0	2.28	0.47	50.0
Diiodotyrosine	6.36	0.90	4.12	19.0	98.6
N-acetyl-tyrosine	10.22	-	4.17*	0.02	1.0
N-acetyl-monoiodotyrosine	8.83	-	4.97*	0.50	18.8
N-acetyl-diiodotyrosine	7.12	-	8.00*	37.5	92.4

* These rates include approximately 4.18 cm^2/KV-hr. for the unbalanced negative charge of the carboxyl group.

moved 4.4 cm. to the cathode by electroendosmosis, the diiodotyrosine migrated a net distance of 6.3 cm. by virtue of the charge on the phenolic hydroxyl. This distance is equivalent to a mobility of 0.90 $cm.^2/Kv\text{-}hr.$

VI.3.2. <u>At pH 8.2 in 0.07M Na barbitone buffer</u>: Tyrosine, monoiodotyrosine and diiodotyrosine (50 μmoles) in 10 μl. of methanol:ammonia were applied separately or together to 5 cm. strips of Whatman 3MM paper.

Electrophoresis was carried out on a high-voltage electrophoresis apparatus where the strips are cooled between flat metal plates insulated by polythene sheeting. The strips which were connected by four thicknesses of filter paper to the electrode vessels were laid horizontally, wetted with the buffer, and blotted; the area around the origin streak was wetted last.

The electrophoresis was carried out at 6 Kv for 1 hr. The papers were dried and as the pH of the buffer was too high for development of the ninhydrin colour, they were sprayed with 1% ninhydrin in acetone, which was 1% with respect to pyridine:acetic acid buffer, pH 5.0. After heating at 80° for 15 min. the positions of the amino acids were revealed.

Table 46 gives the rates of migration of tyrosine, monoiodotyrosine and diiodotyrosine at pH 6.5 and 8.2. The percentage dissociation of the phenolic hydroxyl group calculated from the pK values (Mayberry, Rall and Berman, 1965) indicates that the changes in rate of migration at both pH values can be accounted for in this way.

As with the iodinated tyrosine the rate of migration of the iodinated products of N-acetyl-L-tyrosine increased in proportion to the degree of iodination of the phenolic hydroxyl group (Table 46).

V.4. Chromatography of iodotyrosines in BA:

The R_f values in BA of tyrosine (0.30), monoiodotyrosine (0.57) and diiodotyrosine (0.71) increase as the iodine content of the molecule increases.

V.5. Iodination of peptide A_5

V.5.1. Method: It was known from previous experiments that A_5 comprised 2.8% of the ^{131}I-activity on a peptide map. The present sample of A_5 had been prepared from 21.3 mg. of thyroglobulin of which A_5 would make up 600 μg. A_5 has an apparent molecular weight (from gel filtration) of about 400 and 600 μg. is equivalent to 1.5 μ moles.

As only half of the peptide was iodinated (as a control the remainder was subjected to conditions identical to iodination except that no iodine was used) and as a three-fold molar excess of iodine was used (as determined from iodination of monoiodotyrosine) 2.3 μ mole $^{131}I_2$ was the estimated, and used, amount.

The specific activity of the peptide was calculated as 66 μC/mg. I_2 and the $^{131}I_2$ for iodination was produced with a specific activity higher than this at 300 μC/mg. I_2.

The peptide A_5 was dissolved in methanol:ammonia and two equal samples removed and taken to dryness. Each was dissolved in 100 μl. of 25% aqueous ammonia. To one was added 200 μl. ether containing 2.3 μ moles (580 μg.) of $^{131}I_2$ and to the other 200 μl. ether.

After standing for 1 hr. the samples were evaporated and taken up in methanol:ammonia.

VI.5.2. Electrophoresis of A_5 and iodinated products: Samples of uniodinated A_5, iodinated A_5 and a mixture of the two were electrophoresed in duplicate at pH 6.5 for 5.4 Kv-hr. at 2mAmp/2.5 cm.

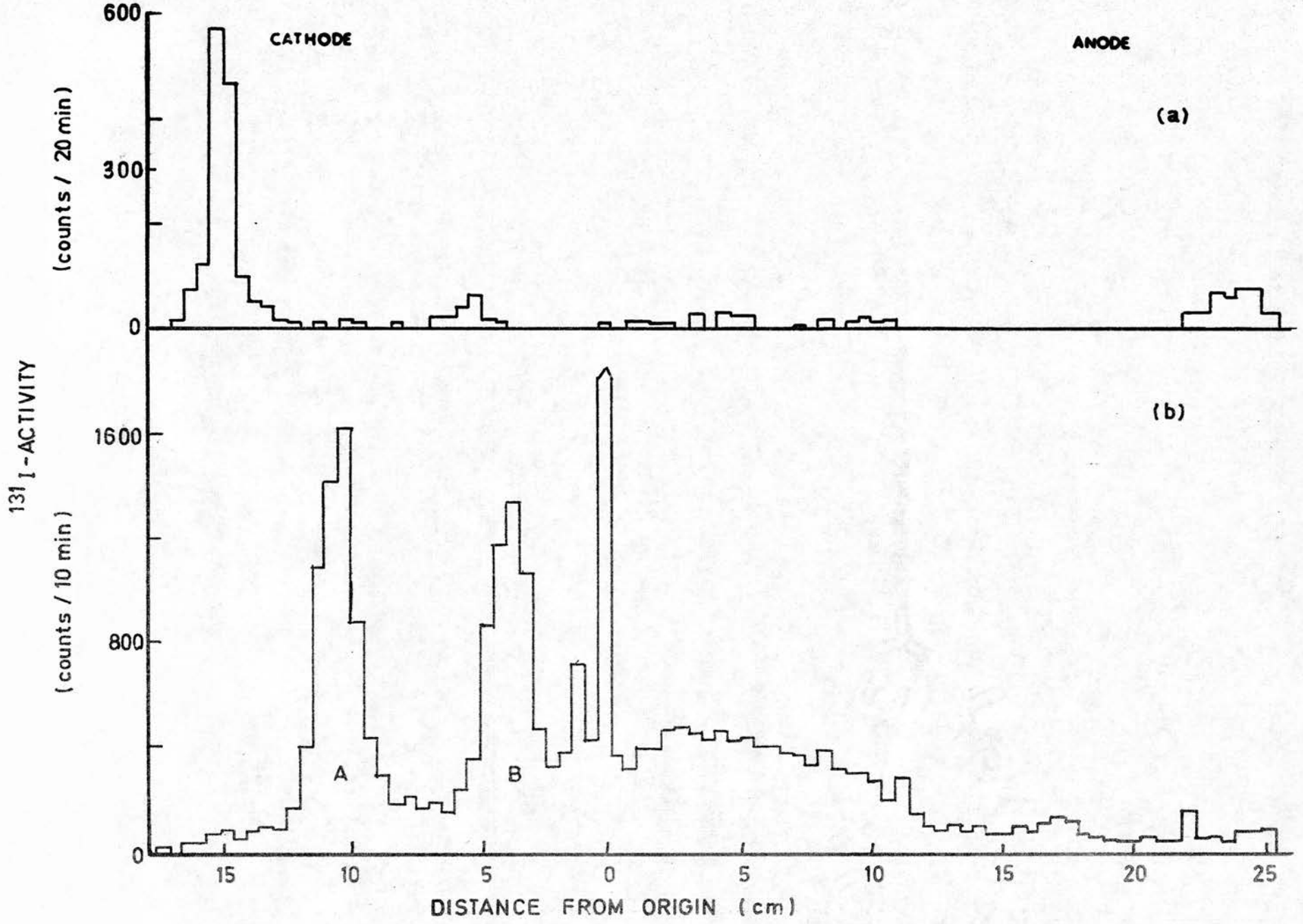


FIGURE 30 ELECTROPHORESIS AT pH 6·5 OF (a) PEPTIDE A_5 AND (b) ITS IODINATION PRODUCTS.

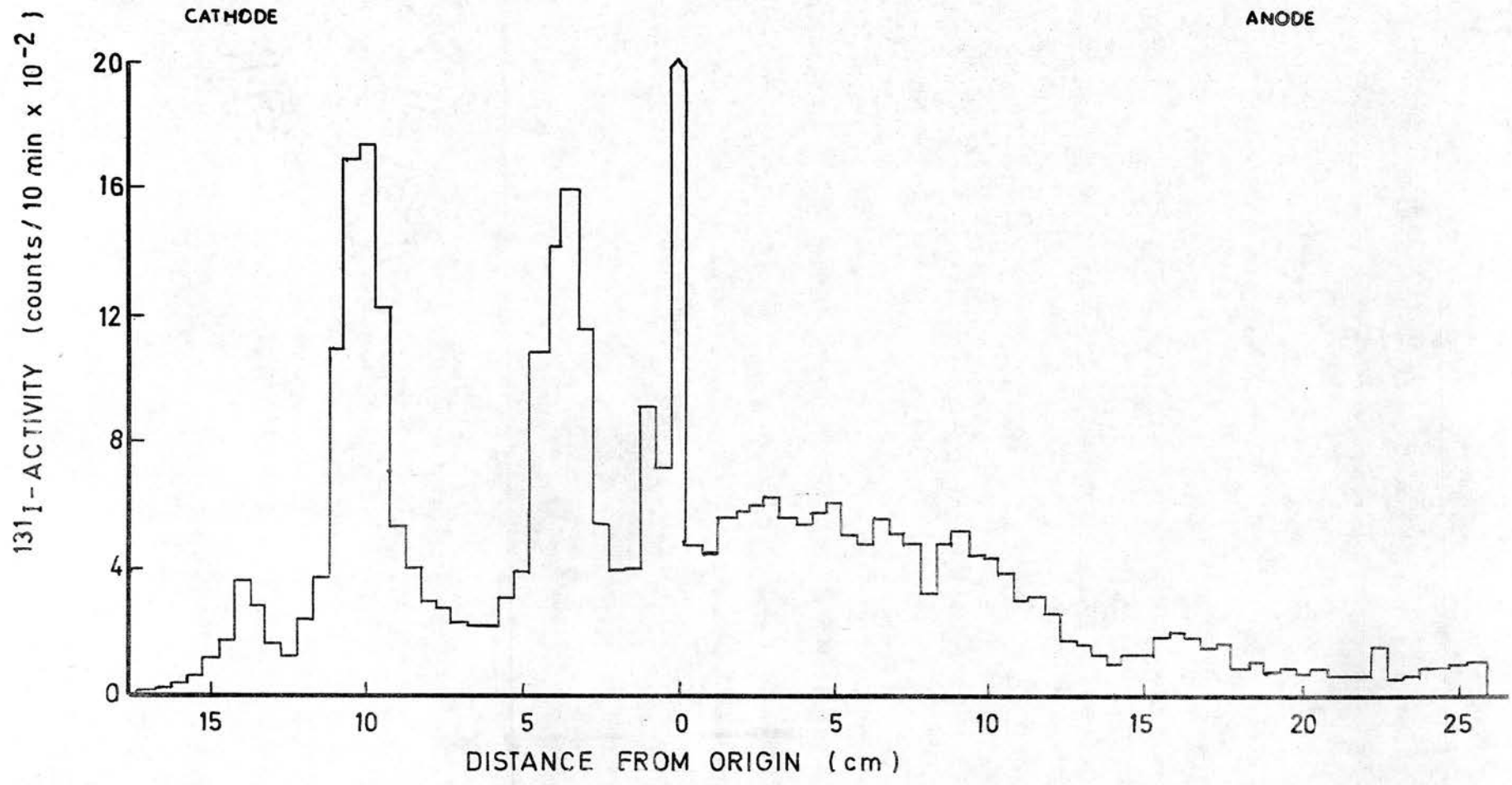


FIGURE 30(c) ELECTROPHORESIS AT pH 6·5 OF A MIXTURE OF PEPTIDE A_5 AND ITS IODINATED PRODUCTS.

After drying, the strips were counted in the Argon Scanner.

A_5 itself appeared as a discrete peak 14.0 and 14.5 cm. (in duplicate) toward the cathode from the origin (Fig. 30,a).

After iodination two distinct peaks were present 9.5 and 10.0 cm. and 3.5 and 3.5 cm. respectively toward the cathode*(Fig. 30,b). There also appeared a large quantity of radioactive material on the origin and a heterogeneous active area toward the anode.

The histogram of the mixed samples showed unchanged A_5 at 14.0 and 15.0 cm., and the two other peaks at 10.0 and 11.0 cm. and 3.5 and 3.5 cm. respectively (Fig. 30,c).

Although the monoiodotyrosine peptide had, on iodination, moved less rapidly to the cathode, as expected, the finding of two peaks required that the products were examined to determine which of peaks was a genuine diiodinated product of A_5.

VI.5.3. Chromatography of A_5: Uniodinated A_5 had an R_f of 0.090 in unidimensional chromatography in BA. During peptide mapping A_5 has an R_f of 0.081. There was a noticeable difference between the R_f values on 5 cm. strips and on peptide maps. The latter had been soaked in pyridine:acetic acid and white spirit and dried at 40°. It was probable that the cellulose fibres had swelled and contained a higher quantity of H_2O as stationary phase.

VI.5.4. Chromatography of iodinated A_5 in BA: The quantity of $^{131}I^-$ present on the chromatograms completely masked any peaks of A_5 or products. Removal of the majority of iodide peak and recounting showed that there was a considerable quantity of polydisperse material whose activity was higher than that of the discrete peaks found by electrophoresis.

VI.5.5. Electrophoresis of A_5 and iodinated products at pH 8.2: Samples of A_5 (20μl.) were added to 5 cm. strips and subjected to

* Peaks 'A' and 'B'

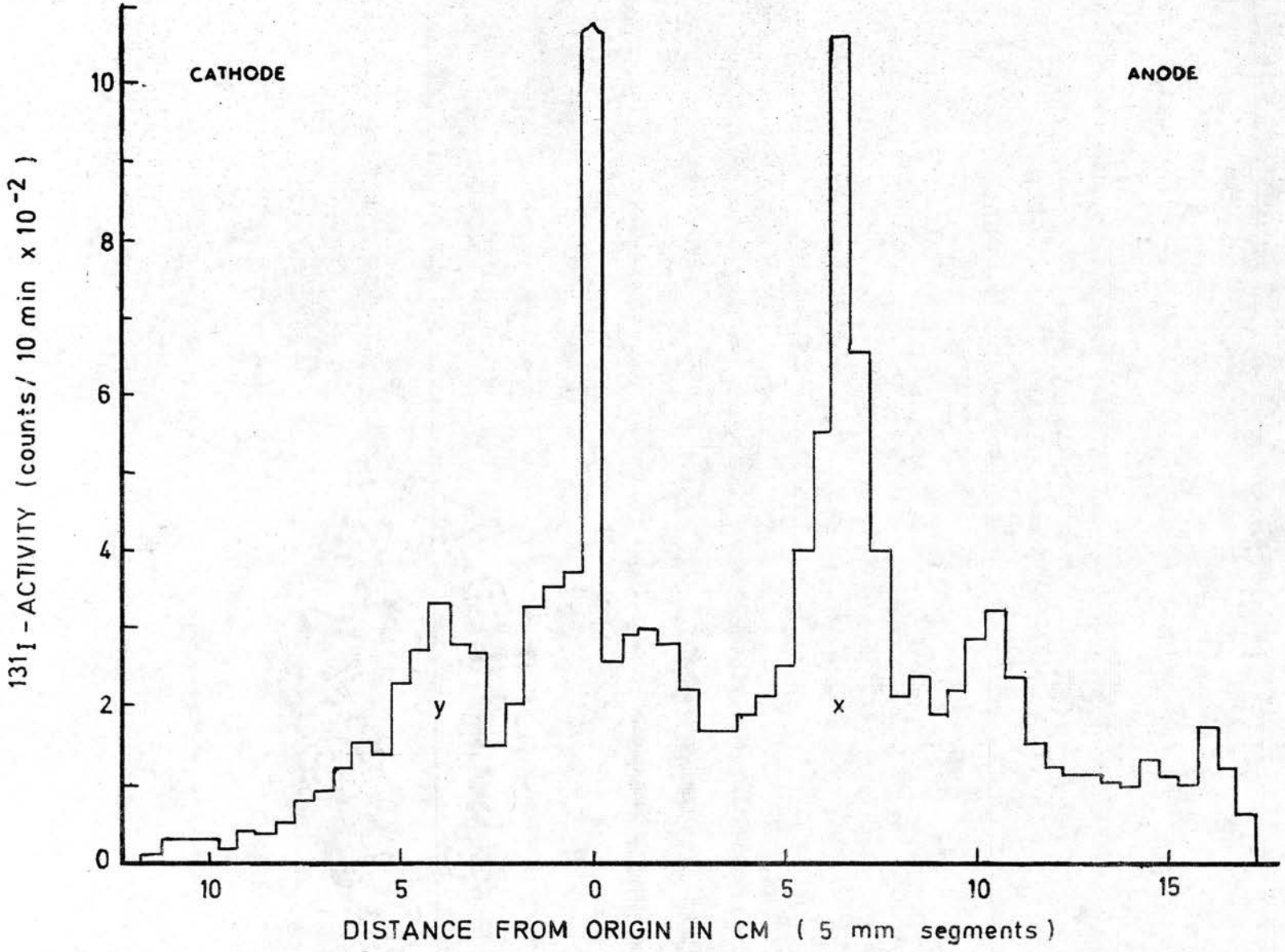


FIGURE 31 ELECTROPHORESIS AT pH 8·2 OF THE IODINATED PRODUCTS OF PEPTIDE A6·

electrophoresis at pH 8.2 for 7.3 Kv-hr. This resulted in A_5 moving 5.0 and 8.0 cm. to the cathode. As mentioned before, the horizontal electrophoresis was not very consistent but as there was only one significant active peak on the strips identification was no difficulty.

Samples (10 μl.) of the iodination product of A_5 were subjected to electrophoresis at pH 8.2 also for 7.3 Kv-hr. Like electrophoresis at pH 6.5 three main peaks were found, one of which was again the origin. Three minor peaks were also present. (Fig. 31).

Of the two remaining major peaks one (y) was in the same position as peak 'B' had been after electrophoresis at pH 6.5 and the other (x) had migrated 6.3 cm. and 7.8 cm. (duplicates) to the anode.

VI.5.6. Chromatography of 'A' and 'B' found after iodination of A_5:

Peaks 'A' and 'B' (Fig. 30,b) were eluted from electrophoretogram of A_5-iodinated products at pH 6.5 and chromatographed in BA and BDA.

In BA 'A' had an R_f of 0.30 and in BDA of 0.22. 'B' on the other hand could not be identified above a general radioactivity on the paper.

The papers were sprayed with $PdCl_2$ and the percentage activities in peptide peaks, iodide and remainder calculated. Although there was a considerable amount of unidentifiable labelled material it was seen that unless 'B' has the same R_f as iodide in both systems, there was virtually none of it present.

'A' on the other hand is the major component in the separation in BDA with only a small amount of deiodination taking place. Chromatography of 'A' in BA again revealed a large peak.

From this it would appear that 'B' is a heterogeneous collection of iodinated material (which at both pH 6.5 and 8.2

migrates to the position of all neutral material moved by electro-endosmosis to the cathode side of the origin) which separates on chromatography into material that cannot be identified as one component.

'A' appears to be the true iodination product of iodination of A_5.

VI.5.7. Further purification of A_5 before iodination: From the data on Table 40 (following p. 108) it was realised that the peptides as isolated from peptide maps contained varying amounts of contaminating radioactive peptides which could be removed by electrophoresis at pH 8.2.

Also N-acetyl-tyrosine (NAT) and N-acetyl-L-monoiodotyrosine (NAMIT) did not separate at pH 6.5, and it was most likely that if A_5 was only the monoiodinated form of a tyrosyl peptide both these forms would migrate together as the percentage dissociation of the phenolic hydroxyl group is only 0.02% and 0.5% for NAT and NAMIT respectively. At pH 8.2, however, the dissociations were 1.0% and 20.0%, giving a separation of about 5 cm./6 Kv-hr.

A_5 was subjected to electrophoresis at pH 8.2 for 7.3 Kv-hr. At this voltage the tyrosine peptide was expected to migrate 18 cm. to the cathode.

After counting, the main part of A_5 was eluted as was the area to the cathode side of A_5, where the tyrosine peptides was expected and where there was a negligible amount of activity. A_5 was again subjected to electrophoresis at pH 6.5, and migrated 15.5 and 15.2 cm. to the cathode as a single discrete peak.

Both A_5 and the possible tyrosine peptide were iodinated as before, subjected to electrophoresis at pH 6.5 and 8.5, and the peaks 'A' and 'B' subjected to chromatography.

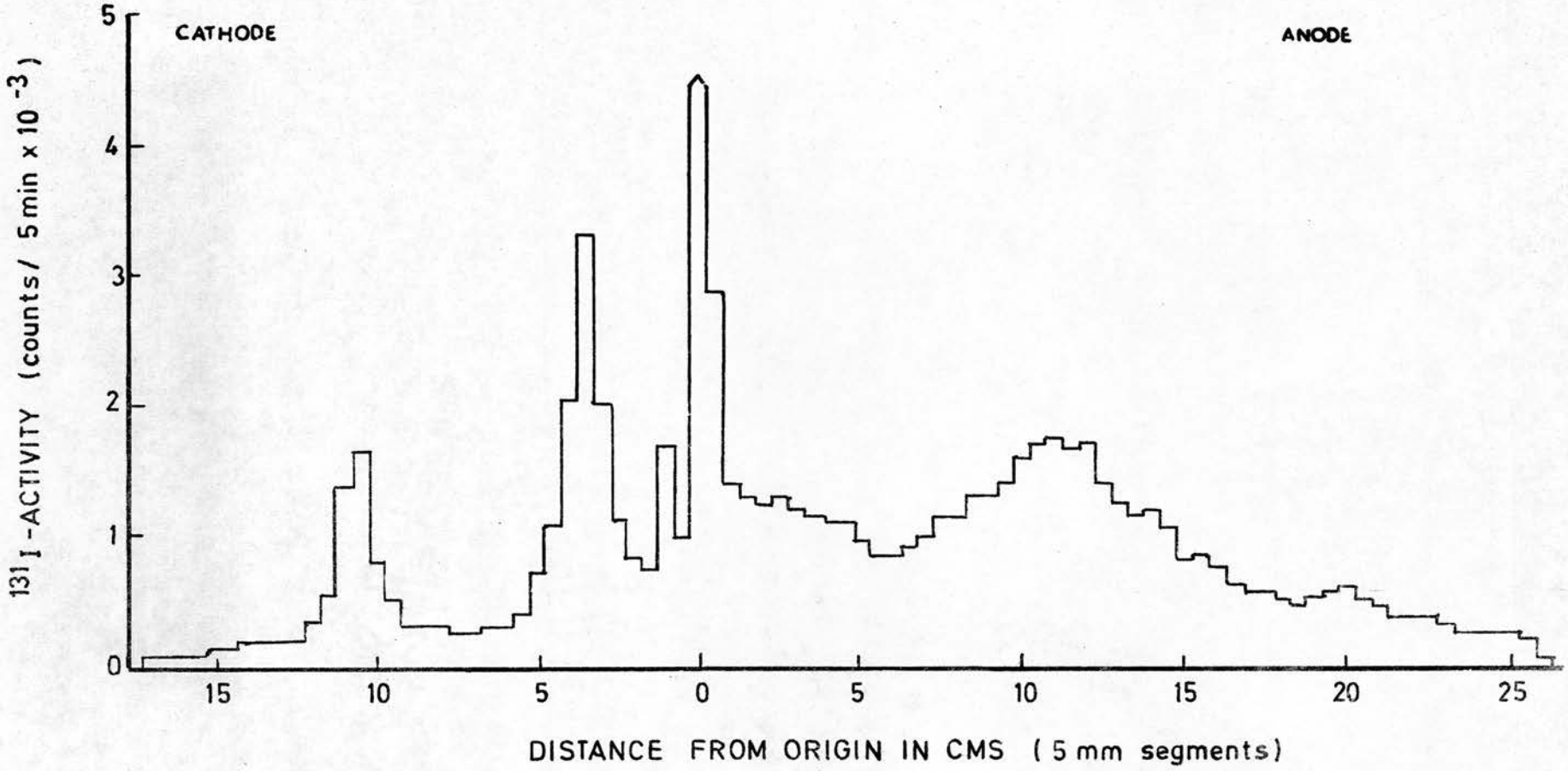


FIGURE 32 ELECTROPHORESIS AT pH 6·5 OF THE IODINATED PRODUCT OF THE MATERIAL THOUGHT TO BE A TYROSINE-CONTAINING PEPTIDE.

Both cases, that is iodination of A_5 and iodination of the material from the area where that tyrosine peptide was expected, resulted in extremely similar pictures of radioactive products (Fig. 32). Electrophoresis of a mixture of the products failed to show any difference between them. Again electrophoresis of a mixture of A_5 with iodinated A_5 showed clean separation between A_5 and peak 'A' of the products.

VI.6. Iodination of monoiodotyrosine to determine peptide quantity

Thyroglobulin hydrolysate from an accurately known weight of thyroglobulin was peptide mapped and the peptides were eluted quantitatively. The peptides A_5, N_8, N_9 and C_{22} were hydrolysed with pronase, the monoiodotyrosine was separated by BA chromatography, detected by counting in the Argon Gas-flow Counter and eluted from the paper. The preparations of monoiodotyrosine were taken to dryness and dissolved in 25% aqueous ammonia, Samples (100μl.) were taken for iodination.

The optimum concentration for the diiodination of monoiodotyrosine was a ratio of 3 moles of $^{131}I_2$ and 1 mole of monoiodotyrosine. Unfortunately the quantity of monoiodotyrosine in the peptides was not accurately known.

If the tyrosyl residue was totally converted to monoiodotyrosine and if thyroglobulin contained no identical sub-units, then 1 molecule of monoiodotyrosine would be found in each peptide per thyroglobulin molecule. However, from earlier results, e.g. double-labelling experiments, it appeared that not all of each available tyrosyl residue was converted to monoiodotyrosine. Allowance was made for monoiodotyrosine comprising only 5% of a certain tyrosyl residue. However, there may be more than 1 mole

of peptide per mole of thyroglobulin.

Each sample of monoiodotyrosine, derived from the peptides A_5, N_8, N_9, or C_{22} from 2 nanomoles of thyroglobulin, was taken up in 100 μl. of 25% aqueous ammonia. To these samples were added 200 μl. of ether containing 0.5, 2.0, 6.0 and 20.0 nanomoles of $^{131}I_2$. The products were chromatographed in BA and counted.

The two smaller amounts of iodine produced no detectable diiodotyrosine. There was, however, an increase in the activity of the monoiodotyrosine area showing that exchange was taking place.

As the quantity of iodine added increased, the area covered by the iodide peak broadened and with the heightened activity the sideways penetration of the γ-radiation into adjacent counters of the Ekco Argon Gas-flow Chromatogram Scanner became more noticeable. For these reasons it was difficult to separate the low activity monoiodotyrosine spot of 200-300 counts/min. from the highly active iodide peak with activities around 10,000 counts/min.

At the level of iodination of 3 moles $^{131}I_2$ per mole thyroglobulin a very small quantity of diiodotyrosine was found with activity hardly larger than twice background.

At the highest level of iodination diiodotyrosine was easily identifiable in all cases, but not at a level suggesting that the conversion from monoiodotyrosine was complete. In spite of the high background in the area of the monoiodotyrosine peak monoiodotyrosine was identified and was more active than that found after iodination with lower levels of $^{131}I_2$ presumably having undergone exchange with highly active added iodine.

The quantities of iodoamino acids present on the chromatogram were very small and this has been shown to lead to poor separation (Plaskett, 1964). Further samples of the same products of

iodination of the monoiodotyrosine from A_5 were chromatographed with 0.5 μmole of diiodotyrosine and monoiodotyrosine carriers.

Scanning of these chromatograms revealed that they were essentially the same as the chromatograms run without marker iodotyrosine, although the separation of the monoiodotyrosine from the iodide peak was improved.

Further samples of monoiodotyrosine, from N_9, again from 2 nanomoles of thyroglobulin, were iodinated with 100 and 200 nanomoles of $^{131}I_2$ respectively. After chromatography in BA, followed by counting, residual monoiodotyrosine was detectable only in the sample iodinated with the lower concentration of iodine. The activity of the diiodotyrosine was slightly greater than double that for the lesser iodination, representing the equilibration with the larger quantity of $^{131}I_2$ and the diiodination of the final quantity of monoiodotyrosine.

Calculation of the quantity of N_9 present is included in Appendix II.

DISCUSSION

The theoretical background to the stages of the research

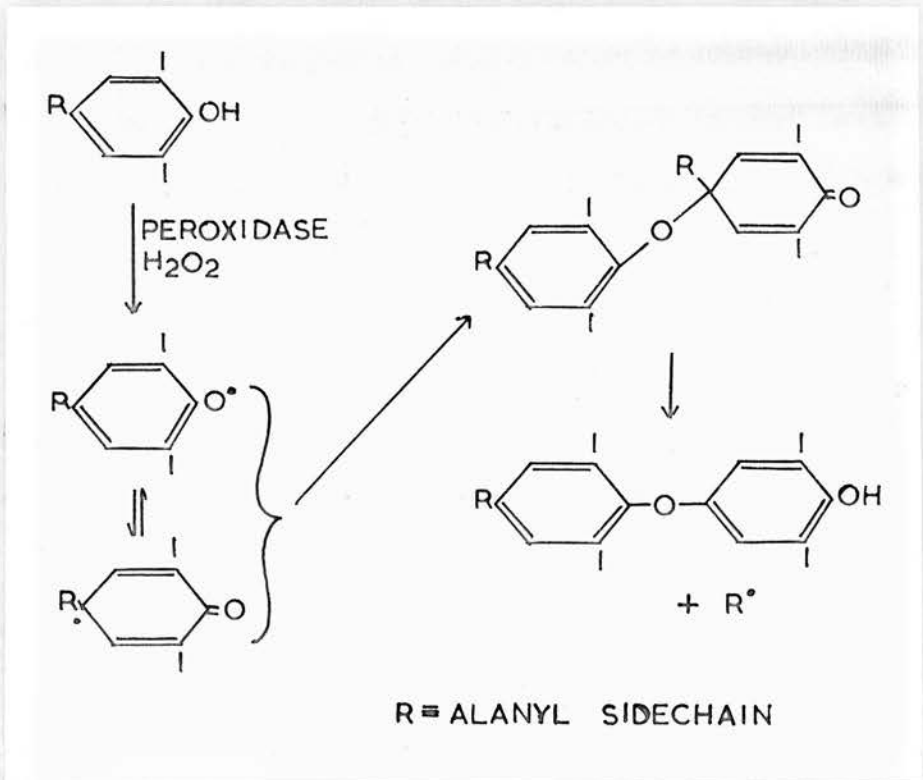
The object of this study was to examine the biosynthesis of thyroid protein and to find how this influenced the synthesis of the thyroid hormone itself. Both the iodination of tyrosyl residues to mono- and diiodotyrosyl residues and the coupling of these to form the thyroid hormones occur in thyroglobulin. The first goal of the research was to establish if iodination was restricted to a few tyrosyl residues and if coupling of pairs of these yielding iodothyronyl residues occurred at certain sites only.

The postulated specificity of iodination (or coupling) may reside either in an enzyme (an iodinase or a coupling enzyme respectively) or in the thyroglobulin itself. There is little evidence in favour of an iodinase using iodide to iodinate specific tyrosyl residues, and a non-specific enzyme system, possibly a peroxidase to oxidise iodide to iodine, is favoured by the balance of information. The majority of thyroidal peroxidases have been isolated from the microsomal fraction of thyroid cells. As histological evidence indicates that 'thyroglobulin' is iodinated only at the apical region of the epithelial cells and perhaps only when it has entered the lumen of the follicle, the role of microsomal peroxidase seems doubtful. It is, however, not inconceivable that the apical membrane itself can oxidise intracellular iodide to extracellular iodine during the passage of the ion across the cellular membrane. Peroxide-dependent iodase activity has been found in a preparation of apical cell membranes by Benabdeljlil,

Michel-Bechet and Lissitsky (1967).

A coupling enzyme to mediate the last stage in iodothyronine synthesis is unlikely (Stanbury, 1967) although model experiments yield higher levels of coupling in mildly oxidising conditions. The <u>in vitro</u> coupling illustrated in synthetic diiodotyrosyl peptides (Pitt-Rivers and James, 1958) or during the iodination of thyroglobulin (van Zyl and Edelhoch, 1967) takes place only in mild alkali under oxidising conditions, the latter often provided either by dissolved oxygen, iodine itself, or by the peroxide:peroxidase: iodine system used for the <u>in vitro</u> iodination of thyroglobulin.

Yamazaki (1958) working with horse radish peroxidase and hydrogen peroxide found that 'oxidogenic' hydrogen donors similar to diiodotyrosine, such as guaiacol, resorcinol and phenol, form free radical intermediates which polymerise very readily. The diiodotyrosyl coupling reaction by this mechanism will have the form below (after Yamazaki, 1958):



This mechanism does not differ materially from that proposed by Johnson and Tewkesbury (1942), discussed in the introduction, except that the production of free radical intermediates is enzyme-induced. More than one peroxidase has been isolated from thyroid tissue and one of these, or a phenol oxidase, may provide the oxidising conditions required *in vivo* for coupling. Against this direct evidence for an oxidase, the model experiment of Pitt-Rivers and James (1958) yielded much more iodotyrosyl material when diiodotyrosine was part of a peptide chain, suggesting that the diiodotyrosyl residues in thyroglobulin are more reactive than the free iodoamino acid. There is evidence (Malan, 1968, and Tang, 1963) that thyroxine is situated in a hydrophobic site in thyroglobulin and as this site would not be expected on the very surface of the protein, the approach of an enzyme may be difficult. Further, there are examples of the isolation of abnormal proteins with very low levels of thyroxine from the thyroids of goitrous sheep (Falconer, 1967) and humans (Murray and McGirr, 1964, and Stanbury, 1967). The writer inclines to the view that the thyroid contains an enzyme system for oxidising iodide to iodine which then iodinates certain tyrosyl residues in thyroglobulin determined by their accessibility or susceptibility to iodine. It is also considered that the coupling of certain pairs of diiodotyrosyl residues to give thyroxine depends primarily on the orientation and juxtaposition of these residues in thyroglobulin, whether the coupling reaction is initiated enzymically or non-enzymically.

If, in fact, the iodination and coupling stages of thyroid hormone biosynthesis are dependent on the configuration of thyroglobulin, it is of prime importance to investigate both the three-dimensional arrangement of the peptide chains and the amino acid

sequence of these. The former has not yet been determined in a protein as large as thyroglobulin, but it was anticipated that the amino acid composition of short peptides could be determined by a radioactive tracer technique.

It was intended to investigate both iodothyronine and iodotyrosine peptides as the actual and potential sites of iodothyronine synthesis. Iodination of specific tyrosyl residues would be confirmed if the number of peptides labelled with $^{131}I_2$ was small in comparison with the total number labelled with ^{14}C-tyrosine. Labelling of all the amino acids in thyroglobulin with ^{14}C-amino acids during synthesis would enable detection of the constituent amino acids of any peptide under investigation after suitable hydrolysis and separation.

From the foregoing, the first stage of the research was to entail a study of the specificity of iodination, followed by isolation of those peptides where iodination and coupling had occurred and determination of their constituent amino acids. Two approaches were proposed to achieve these objectives. The first was separation of the protein synthesis and iodination stages. Separation of the two stages *in vivo* has been postulated both on kinetic grounds (Nadler, 1965, and Wollman, 1965), and because inhibition of protein synthesis does not affect iodination or *vice versa* (Maloof *et al.*, 1964). The iodination reaction appears at the apices of the epithelial cells (Nadler, 1965) and is not associated with the microsomal fraction of the cells. Isolation of a cell-free protein synthesising system would separate this process completely from the iodination reaction. The uniodinated protein labelled with ^{14}C-tyrosine produced by such a cell-free system was to be subjected to *in vitro* iodination, either chemically or by a cell-free system from thyroid.

Separation of the ^{14}C-tyrosine-containing peptides would reveal which of these contained iodine and hence might be implicated in iodothyronine synthesis. The second approach was the simultaneous labelling of thyroglobulin with ^{14}C-tyrosine and $^{131}I_2$ using *in vitro* surviving tissue preparations, and, as before, isolating the peptides containing both labels with determination of their constituent amino acids.

The identification of the very small number of peptides in which thyroxine arises in thyroglobulin was expected to be eased using the former approach. The uniodinated protein was to be iodinated with very small but increasing quantities of $^{131}I_2$ so that the most reactive tyrosyl residues would be in effect titrated with iodine. The second approach using surviving tissue preparations suffered from the disadvantage that there was little control over the iodination step. It was hoped, however, that incubations of the preparation with $^{131}I^-$ for different times might, if resulting in a changing pattern of iodination of tyrosyl residues, lead to the same information as the first method. One advantage of the second method was that the iodination might resemble closely that occurring *in vivo*.

A cell free system which would actively incorporate ^{14}C-amino acids into protein was successfully isolated from thyroid. This preparation, although more active than similar ones reported (see, for instance, Morais and Goldberg, 1967, whose preparation had a maximum incorporation into protein of only 0.2% of the given ^{14}C-amino acid/mg. of isolated protein) did not produce a protein sufficiently labelled for the proposed later investigations. During this work on the thyroidal cell free system several interesting differences between this system and others from different sources

came to light. These will be discussed more fully below.

As it had not proved possible to isolate a sufficiently highly labelled non-iodinated thyroglobulin by the cell free system the point of attack was shifted to the use of the *in vitro* surviving tissue preparations. Two *in vitro* preparations were investigated, namely surviving rat thyroid-trachea preparations and surviving sheep thyroid slices, and the latter was found to incorporate ^{14}C-amino acids into thyroglobulin very actively. This preparation also took up iodine rapidly, resulting in a highly labelled ^{131}I-thyroglobulin. This second approach proved to be very successful, and resulted in thyroglobulin highly labelled with ^{14}C-amino acids and $^{131}I_2$. After separation of the labelled peptides, it was found that, in contrast to about 90 ^{14}C-tyrosine labelled peptides, there were only about 40 iodotyrosyl peptides, and of these about 12 were of especially high activity. This finding indicated that the iodination of tyrosyl residues in sheep thyroglobulin is at least partly specific, confirming the results of Malan (1968), van Zyl and Edelhoch (1967), and Edelhoch and Perlman (1968). It was at this stage that the determination of the amino acids comprising the specifically labelled iodotyrosyl residues was to be carried out. This was done (Appendix III) on a non-iodinated peptide whose ^{14}C-labelled amino acids were detected after two-dimensional separation following acid hydrolysis. Unfortunately, none of the peptides from the doubly labelled thyroglobulin could be shown to contain both $^{131}I_2$ and detectable ^{14}C-tyrosine, even by autoradiography of the same peptide map for the two isotopes separately. This, at first sight rather surprising result, which is discussed below, completely precluded the determination of the amino acid composition of the peptide chain adjoining the iodinated tyrosyl

residues.

Up to this stage of the work the emphasis had been on isolating the iodotyrosyl peptides which were expected to be the precursors of the iodothyronyl peptides in thyroglobulin and on determining their amino acid composition. As a peptide containing both ^{14}C-tyrosine and $^{131}I_2$ had not been identified, the investigation of the iodotyrosyl peptides themselves was extended.

A certain amount of work had been done to determine the molecular size of the iodotyrosyl peptides so that if these peptides proved to be still large, they might be further enzymically split and thus allow easier identification of the amino acids in the protein, including the iodotyrosyl residue. For convenience, this earlier work is discussed together with the later work on investigation of the iodotyrosyl residues. It was evident that α-chymotryptic hydrolysis degraded thyroglobulin so that the majority of the iodotyrosyl residues were attached to peptides of low molecular weight. Purification of some of these peptides by electrophoresis revealed that they contained only one iodotyrosyl residue. This was important as it confirmed the specificity of iodination of thyroglobulin. If the 'peptide' as detected by autoradiography of peptide maps had contained two or more iodotyrosyl residues, this would have increased the total number of iodinated tyrosyl residues toward the total number of tyrosyl residues in thyroglobulin.

It had been found that the thyroxine content of the sheep thyroglobulin iodinated <u>in vitro</u> was low and the coupling of diiodotyrosyl peptides was virtually non-existent. Determination of the iodotyrosyl content of the iodopeptides would allow the identification of these peptides with a high diiodotyrosyl content which were likely to be the <u>in vivo</u> precursors of thyroxine.

At this stage of the study it was becoming possible to investigate to some extent the iodination stage in the hormone biosynthesis. From several studies it has been shown that tyrosyl residues are first monoiodinated and subsequently diiodinated (see, for instance, Fig. 29, following p. 121). It is, however, not clear whether a given tyrosyl residue has the intrinsic property or being iodinated either to the mono- or diiodinated form, although the latter will presumably, during iodination, go through two sequential iodination stages as the likelihood of a simultaneous attack by two iodine molecules is doubtful. It appears from iodination studies (Li, 1945) that monoiodotyrosyl is more susceptible to further iodination than is tyrosine itself (although Mayberry et al., 1965, found the opposite to be true for the iodination of free acetyl tyrosine) perhaps because of the lowering of the pK value of the phenolic hydroxyl from 10.13 to 8.20 for the free amino acids. Thus, if monoiodotyrosyl peptides are found (and they are more prevalent than diiodotyrosyl peptides) they may have remained in such a state because of an intrinsic property of that particular 'site', such as a peculiarly low concentration of iodine near the particular residue. Moreover, as it is known that low iodine in the diet or during in vitro iodination of thyroglobulin both produce lower levels of monoiodotyrosyl and concentrations of diiodotyrosyl and thyroxine relatively even lower, it is feasible that a low concentration of iodine at any site will lead to a low level of monoiodination, and the likelihood of no diiodotyrosyl or thyroxine. In this line of argument certain sites will have only low levels of monoiodotyrosyl. In other sites exposed to greater iodine concentrations there will be an increasing conversion of monoiodotyrosine to diiodotyrosine. If this is so, the

monoiodotyrosyl peptides from thyroglobulin should have less than half the ^{131}I-activities of the diiodotyrosyl peptides, allowing for the two labelled atoms in diiodotyrosine.

This theory has several pieces of circumstantial evidence to support it. The evidence cited above shows that low iodine leads to a predominance of the less iodinated amino acid. The hypothesis would explain the iodination of pre-formed 19S protein in thyroid slices which takes place in a protein already iodinated and 'matured' with formation of thyroxine in vivo before the sacrifice of the animal. This continuing iodination indicates that as iodine becomes available it is incorporated into monoiodotyrosyl and diiodotyrosyl residues. A further piece of evidence was supplied (Fig. 29, following p. 121), by the increasing ratio of diiodotyrosyl to monoiodotyrosyl with increasing times of incubation with iodine. This increase is exactly that expected as the level of $^{131}I^-$ in the thyroid slices increases leading to more diiodination.

An alternative to this theory of regulation of iodine supply is that electronic or steric interactions of the phenyl ring with surrounding groups would be expected to influence the extent of iodination of each tyrosyl residue. Evidence for this has been supplied by Edelhoch (1962) who found that only 40% of tyrosyl groups could be iodinated chemically without a change in the configuration of thyroglobulin. That the other groups are not iodinated in vivo indicates that they are not susceptible to attack by iodine. Among the group of tyrosyl residues open to iodination by this theory it is possible that, under restricting conditions, some tyrosyl residues could be monoiodinated to a high level of ^{131}I-activity greater than that of some diiodotyrosyl residues. This situation is not, of course, possible by the former hypothesis. Determination

of the iodoamino acid in each peptide and the ^{131}I-activity of the peptides can be expected to demonstrate which of the above ideas is tenable.

Up to this point the mono- or diiodination of tyrosyl residues have been discussed as mutually exclusive events. It is, however, also probable that tyrosyl residues will be only partially converted to monoiodotyrosyl, or that both the monoiodotyrosyl and diiodotyrosyl forms of a tyrosyl residue will co-exist. On the autoradiograms of the ^{131}I-peptide maps the iodotyrosyl peptides were close to each other and it appeared possible that the change in mobility induced in a peptide by changes in iodination level of the iodotyrosyl residue might be sufficient to separate the two forms of the one residue. That the two or three forms of a tyrosyl residue might exist was possibly implied by the lack of peptides labelled with both ^{14}C-tyrosine and $^{131}I_2$ which would occur if only a small proportion of any tyrosyl residue was iodinated leaving the bulk of the ^{14}C-tyrosine label as uniodinated peptide and the remaining small proportion of ^{14}C-tyrosyl residue iodinated.

Two methods were employed to yield information on this point. One indirect way was to study the changes in mobility of tyrosine and N-acetyl-tyrosine at two pH values and during chromatography in BA after mono- and diiodination. N-acetyl-tyrosine was thought to be analogous to the iodotyrosyl peptides (by virtue of its blocked α-amino group and free carboxyl group), so that the quantitative changes in its mobility on iodination were expected to indicate how the iodopeptides would behave after diiodination, and whether the changed mobility of the iodinated peptide was similar to an already-identified diiodotyrosyl peptide. The second direct method consisted of iodinating a monoiodotyrosyl peptide to determine

whether the product was identifiable with an already-found diiodotyrosyl peptide. The outcome of these experiments is discussed below.

The above discussion illustrates the rationale behind the various stages of the research and shows the type of results expected from the a priori hypotheses on the questions under study. The sections below discuss the results and conclusions from the research and determines where these agree or differ from the above hypotheses.

The cell free protein synthesis preparations

The cell free system from rat liver used in the preliminary experiments was found to be similar in all respects tested with other rat liver systems reported in the literature. (Zamecnik and Keller, 1954, and Korner, 1962). Both the homogenate and the isolated microsomal preparations remained active in incorporating ^{14}C-leucine for a considerable period of time.

One of the main criteria of genuine protein synthesis (Hoagland, 1960) is that the incorporation be energy dependent. The rat liver cell free system was inhibited by more than 80% in the absence of an ATP-generating system; an effect not altered by incubations under different gas phases.

Each of the cell free systems from rat thyroid incorporated ^{14}C-leucine and some proved to be more active than the corresponding liver preparations.

As with several other cell free systems, isolation of the microsomal fraction after incubation revealed that practically all

the label was attached to the ribosomes. Although Korner (1962) demonstrated the release of labelled albumin from rat liver ribosomes, other workers found that protein material could only be removed by further treatment such as ultrasonication or digestion with ribonuclease (Campbell, Greengard and Kernot, 1960). Conditions for protein release appear to be critical (Lamfrom, 1961, and Hultin, Leon and Cerasi, 1961). Nunez, Mauchamp, Macchia, Jerusalami and Roche, (1965) found, after digitonin extraction of labelled particles from a thyroid cell free system and sucrose gradient centrifugation, that only material with low S values was present.

In the absence of released labelled thyroglobulin it is impossible to say that de novo protein synthesis was occurring. Even release of completed protein may indicate only the finishing of an already-started molecule. The product of such a system will not be uniformly labelled, but will have more ^{14}C-amino acid toward the C-terminal end of the peptide chain. Non-uniform labelling of this type would have made the interpretation of peptide maps of this protein very difficult.

There are, however, several interesting differences between the cell free system from rat thyroid and other cell free systems.

Ribosomes are extremely sensitive to the magnesium concentration, and dissociate into two inactive fragments at levels below 5mM. The activity of the rat thyroid system was virtually independent of magnesium concentration over a range from 0 to 6 mM.

Even more interesting was the lack of energy-dependence exhibited by some of the preparations, and the effect that the gas phase had upon the incorporation. The stimulation of incorporation under oxygen and the marked inhibition under nitrogen suggest that the system being studied is not simply a microsomal one.

The conditions for the separation of sub cellular fractions have until recently been based mainly on results from rat liver, other similar tissues, and also from easily disrupted tissues, such as rabbit reticulocytes (Allen and Schweet, 1962). The standard centrifugation procedures for rat liver can isolate preparations of mitochondria, microsomes or ribosomes that under the electron microscope are found to be almost entirely pure. These conditions do not hold for thyroid tissue. Ekholm (1961) has shown by electron-microscopy that the thyroid 'mitochondrial' preparation contains a large proportion of microsomes. Nunez et al. (1965) isolated, from sheep thyroid, two sub cellular fractions sedimenting between 700 g and 105,000 g and between 15,000 g and 105,000 g and found that the former mixed preparation gave the most reproducible incorporations.

This indicates either that the rough endoplasmic membranes of thyroid tissue are less disrupted and hence sediment with the mitochondria, or that the two fractions are associated, possibly artefactually, after homogenization. Confirmation of this theory is indicated by the uptake of ^{14}C-leucine into the sub cellular fractions of sheep thyroid slices (Table 19, following p. 54) where the 'mitochondrial' fractions are more highly labelled than the microsomal fraction. Also, the microsome-cell sap preparations from rat thyroid exhibit some characteristics of a mitochondrial preparation, namely, stimulation under oxygen and occasional inhibition in the presence of ATP.

While it is well-known that mitochondria carry out protein synthesis (Halder, Freeman and Work, 1966), and that this activity requires ATP, it is unlikely that fragmented mitochondria retain an oxidative phosphorylation system active enough to synthesize ATP in the presence of oxygen and endogenous substrate.

Soffer and Mendelsohn (1966) found that ^{14}C-arginine is incorporated by two systems from sheep thyroid: one requiring ribosomes and supernatant, and the other only the supernatant fraction. The latter system requires ATP and supplementary soluble RNA, but is not inhibited by puromycin or lack of Mg^{++}. A second system (Soffer, 1967) is described where some extracts of sheep thyroid mitochondria incorporated ^{14}C-glutamic acid into hot trichloracetic acid-insoluble, protease-digestible material in the absence of ribosomes. Pyruvic acid is required. ^{14}C-α-ketoglutarate is rapidly incorporated with no requirement for pyruvate. The product is rendered acid soluble by pronase and the activity is heat labile. The reaction does not require ribosomes, Mg^{2+}, or GTP, is not inhibited by puromycin or ribonuclease, and is absent from thyroid cytoplasm.

Similarily with rat thyroid, incubation of the mitochondrial, microsomal, and supernatant fractions separately with ^{14}C-leucine showed incorporation of the amino acid into all fractions - the mitochondrial fraction being the most active and the microsomal the least.

The incorporation by the cell sap in the absence of the microsomal fraction indicated that the ^{14}C-leucine was probably undergoing some form of transfer reaction, possibly mediated by the activating enzymes and the transfer RNA. The ^{14}C-leucine was not removed from the trichloracetic acid-insoluble material during purification. The amino acid was released only after pancreatin hydrolysis, and identified by chromatography in BA. All ^{14}C-activity was found in the leucine peak.

The incorporation of leucine by the rat thyroid system in the presence of increasing quantities of ^{12}C-leucine remained constant

at a level of approximately 2 mμg./mg. of protein (Table 9c, following p. 34). This may indicate a limited number of sites, such as the C-terminals of uncompleted peptides, for addition of amino acids. This is in direct contrast to the proportional increase in incorporation with increasing concentration of ^{12}C-leucine added to rat thyroid-tracheae preparations (Table 15, following p. 47).

Although the unique features of the isolated cell free system might have repaid further investigation, the object of its isolation was the production of highly labelled thyroglobulin.

Not only was the protein isolated from the above expeiments small in quantity, but in view of the unusual properties of the system, it was doubtful whether the protein isolated was uniformly labelled, or, indeed, whether the label was in the positions expected by *in vivo* synthesis.

Peptide mapping and the iodotyrosyl peptides

The peptide mapping of doubly labelled thyroglobulin and the uptake of iodine into individual peptides have yielded what are, perhaps, the most interesting results of the research.

The iodine metabolism of the thyroid slice preparation, excluding the coupling stage, appeared to proceed in a way little different from that *in vivo*. Iodine is taken up very rapidly into thyroid slices and organically bound. The sliced sheep thyroid system synthesizes less than physiological amounts of thyroxine (see, for instance, Nunez, Mauchamp and Roche, 1964) and the monoiodotyrosyl to diiodotyrosyl ratio is higher than *in vivo*. Lissitsky (1966) found 9 molecules of monoiodotyrosyl and 6 of diiodotyrosyl per mole of sheep thyroglobulin and hence a ratio of ^{131}I-activities for

these after isotopic equilibration of 0.75. Mauchamp et al. (1965) found an in vivo ratio of 0.3, and in vitro, after incubations of 20-30 hr., of 2. In the present work the monoiodotyrosyl to diiodotyrosyl ratio was at this latter value only for incubations of 1 min. and fell to 0.9 after 8 hr.

This change in ratio has been discussed in the General Introduction (p. 13) and is thought to be caused by a two-pool system for iodotyrosines in thyroglobulin where the pool sizes and turnover rates are influenced by the previous history of the animal. This may be the situation here, or simply that under non-physiological conditions the conversion of monoiodotyrosine to diiodotyrosine is less rapid, making the precursor-product relationship more obvious. In some in vitro preparations, notably thyroid cell tissue culture, the cells may lose the ability to iodinate tyrosyl residues to a stage further than monoiodotyrosine.

The chymotryptic peptides separated by peptide mapping were found to be of small size - over 70% of the ^{131}I-activity in the hydrolysate was present in material with an apparent molecular weight less than 1,000. Purification of some of these peptides showed that they contained either monoiodotyrosine or diiodotyrosine but not both.

Different preparations of ^{131}I-thyroglobulin contained similar but not identical percentages of the total iodine in the individual peptides. This effect was not great enough to alter the pattern of peptide mapping nor to change the identity of the peptides and probably stems from past history of the sheep as the incubation conditions were standard, and the quantity of carrier-free $^{131}I^-$ added was approximately 10 nanomoles/10 ml.

Using a single batch of slices incubated with $^{131}I^-$ for

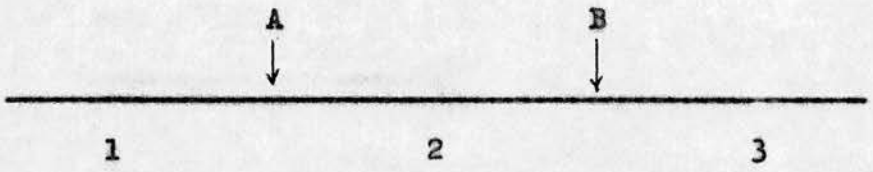
increasing time, the total activity of the thyroglobulin rose from 13.6 mμC/mg. after 20 min., through 62.0 mμC/mg. at 1 hr. to 104 mμC/mg. after 4 hr. 40 min. In spite of this eight-fold rise in activity, virtually all the peptides maintained a constant percentage of the total activity (Table 44, following p. 121).

During the discussion of tyrosyl residue iodination it was suggested that restricted access of iodine to certain residues would result in their having a low content of ^{131}I-monoiodotyrosine. Other sites less restricted would contain as well ^{131}I-diiodotyrosine at considerably higher activity. From Table 44, where some of the activities of the most active peptides are listed, it is evident that some of the monoiodotyrosine peptides contain more $^{131}I_2$ than some of the diiodotyrosine peptides. This result is incompatible with the theory that locally restricted access of iodine results in a certain tyrosyl residue remaining in the monoiodinated form, bearing in mind that monoiodotyrosine is probably more easily iodinated than tyrosine. The result is, however, in agreement with the alternative proposal that the nature of the electronic and steric environment of a tyrosyl residue determines whether mono- or diiodination occurs. In physical terms the situation may be visualized as the phenyl ring lying close to a cleft or sunken area of the protein surface. Iodination will cause the phenyl ring to rotate about the bond <u>para</u> to the hydroxyl group so that the bulky iodine atom is accorded the maximum amount of space. This necessitates that the other side of the ring swings into the cleft or otherwise restricted environment so that the other <u>ortho</u> position is no longer available for iodination. An extension of this theory is obviously the phenyl ring which is totally buried in thyroglobulin, probably playing a role in the

structure of the protein, and which is not at all accessible to iodination.

If this is the complete picture, it would be expected that the accessible tyrosyl residues would be either completely mono- or diiodinated. This situation would result in two groups of peptides - one, the monoiodotyrosyl peptides, with exactly half the ^{131}I-activity of the other, the diiodotyrosyl. This was not found (Table 44) indicating that the iodination of at least some of certain tyrosyl residues takes place to only a limited extent, perhaps reflecting the differential susceptibility of some phenyl rings to iodination, an effect depending on factors such as the proportion of the rings in the phenolate ion form. This effect may be influenced by changes in the shape of thyroglobulin as it becomes more highly iodinated. The pK values for free tyrosine and its iodinated forms are 10.13, 8.12 and 6.36, and for N-acetyl-L-tyrosine and its iodinated forms 10.20, 8.80 and 7.16 (Mayberry et al, 1965), and the pK values of the corresponding residues in thyroglobulin will probably be higher still. Assuming a cellular or luminal pH of 7.4, iodination will result in the ionization of the phenolic hydroxyl group from virtually zero to 4% and 63% respectively. This increase in point charge may be expected to alter the protein configuration, in a manner analogous to that in haemoglobin during sequential oxygenation of the four haem groups, resulting in decreased accessibility of other tyrosyl residues. A further factor requires to be considered, namely, that α-chymotrypsin during digestion of lengths of peptide chain containing two or more susceptible bonds, may, by hydrolysing one of these bonds, render the other bond less liable to attack. Thus hydrolysis by α-chymotrypsin at A and B (see below) will yield the expected three

peptides (1, 2 and 3) but may also result in significant proportions



of peptides 1-2 and 2-3. A factor or combination of factors of this kind in thyroglobulin may be responsible for the range of ^{131}I-activities of the iodotyrosyl peptides, especially the 30 or so peptides which contain less than 1% of the total ^{131}I-activity each.

The results of the chemical iodination studies which were undertaken in Section VI have been discussed (pp. 145-6). Attempts were made to correlate the electrophoretic mobilities of A_5 and its R_f values before and after iodination with those of diiodotyrosine peptides A_6 and A_8 which are found close to A_5 on peptide maps. The iodinated product of A_5 could not be shown by these indirect methods to be identical with A_6 or A_8. It was suggested, however, that the iodotyrosyl peptide A_5 is accompanied by its uniodinated tyrosyl form and that the two together constitute 4 moles of peptide per mole of thyroglobulin, a result in agreement with the postulated number of identical sub-units in thyroglobulin. The second finding (Appendix II), that two single monoiodotyrosine peptides, isolated from the mixed peptide, N_9, are each present at approximately 5 moles/mole thyroglobulin, appears to confirm that both these particular tyrosyl residues are fully monoiodinated.

The picture at this stage of the work was that, of the many tyrosyl residues, only a certain number, possibly about 30, could be iodinated and steric hindrance restricted diiodination to only a

few of these. There was no reason to suppose that either mono- or diiodination of the residues was complete. This picture was supported by the finding that a proportion of a tyrosyl residue was present in both the uniodinated and monoiodinated forms, although the diiodinated form of the same peptide was not detected.

Turning to the peptide maps of the ^{14}C-tyrosine labelled thyroglobulin, 91 peptides were detected. The activities of these peptides were reinforced, and further ones detected, after labelling of other amino acids. In spite of this, comparison of the autoradiograms of the ^{131}I- and ^{14}C-activities from the same chromatogram revealed that none, or virtually none, of the labelled peptides contained both isotopes. This was surprising as both isotopes are incorporated rapidly for several hours. This finding, however, confirms the finding of Mauchamp et al. (1965) who reported that incubation of sheep thyroid slices with ^{14}C-tyrosine and $^{125}I^-$ produced iodotyrosines labelled only with $^{125}I_2$. By sucrose gradient centrifugation they found the ^{14}C-tyrosine labelled thyroglobulin had a sedimentation constant of 17S and that this was increased to 19S following chemical iodination. This latter protein yielded ^{14}C-monoiodotyrosine and ^{14}C-diiodotyrosine.

They also showed that ^{125}I-thyroglobulin was heterogeneous - the peak of absorbance at 210 mμ(19S) sedimenting slightly faster than the peak of ^{125}I-activity (18.4S). This result was also confirmed (Fig. 19, following p. 78) although the lighter material was found to be 17S.

Mauchamp et al. (1965) believed that the 17S protein was the newly synthesized non-iodinated precursor of the 'more or less halogenated family defined as thyroglobulin'. Incubation of slices with PTU, ClO_4^- and ^{14}C-tyrosine yields 17S protein which can then be

chemically iodinated to a mono- and diiodotyrosine-containing 19S protein. Mauchamp et al. (1965) thought iodination took place on the preformed 19S material in the colloid, hence separating the iodination stage from the intracellular stages. It appears that this non-iodinated material is mixed with the older iodinated material during homogenization, but under physiological conditions will be transported to the iodination sites and thence to the colloid.

However, the present work shows that incubation of slices with ^{14}C-tyrosine alone leads to a large release of ^{14}C-protein into the cell sap and, at a later time, into the medium surrounding the slices. It seems paradoxical that iodination should continue for at least 8 hr., and very rapidly at early times, at the same time that up to 50% of ^{14}C-label incorporated is present in the cell sap and 15% of the label is released into the incubation medium without synthesis of ^{14}C-monoiodotyrosine and ^{14}C-diiodotyrosine. The sites of iodination and protein synthesis must be separated rigidly.

The process of maturation of 17S protein to the 19S form is complex and appears to involve not only iodination, as witnessed by the production of ^{131}I-17S protein, but probably the coupling of diiodotyrosine residues to give thyroxine. In the slice preparation where very little coupling takes place, there is iodination of preformed thyroxine-containing 19S protein. Iodination of 17S protein without maturation results, probably, in a range of iodinated ^{14}C-labelled proteins all with a partially uncoupled structure, ranging in sedimentation constants from 17S to 19S. The basic two-pool system for the iodotyrosines (Plaskett et al., 1963,a) is based on the heterogeneity of thyroglobulin hydrolysis by the thyroidal proteases. A molecule which has 'matured', that is, now has a more compact structure and contains one or more molecules of thyroxine,

is less susceptible to hydrolysis. The other molecules will be hydrolysed and the iodotyrosines deiodinated. On this basis only iodine incorporated into preformed 19S protein will not be subject to the recycling process and these iodotyrosyl residues will not have been labelled with ^{14}C-tyrosine.

It still remains to explain the presence of ^{14}C-labelled thyroglobulin and the 17S ^{131}I-thyroglobulin. The explanation probably resides in the slice preparation itself. Salvatore et al. (1965) found, from three sheep, that the follicles ranged in diameter from 52-184μ, 35-84μ and 22-58μ. The slices used in this work were 0.25-0.3 mm. thick and were probably between 3-7 follicles thick. Approximately half the follicles would be open to the medium and a considerable number of the remainder damaged, leading to leakage of colloid. Thus the ^{14}C-tyrosine-labelled thyroglobulin which passes out of the epithelial cells of damaged follicles may escape iodination, which is thought to occur on the extracellular side of the apical region of the cells, and pass to the medium. In undamaged follicles where iodination can occur this protein will eventually be reabsorbed and hydrolysed. The majority of ^{14}C-thyroglobulin after 8 hr. incubation is still in the cell sap (Fig. 7, following p. 53) and the above argument affects only a minority of the molecules.

The small quantity of 17S ^{131}I-thyroglobulin probably arises from that produced in relatively undamaged follicles and represents that pool of newly synthesised protein which is awaiting either maturation or re-entry into the epithelial cells and consequent hydrolysis. The total quantity of 17S protein by absorption at 210 mμ(Fig. 19, following p. 78) is very small and was not obvious

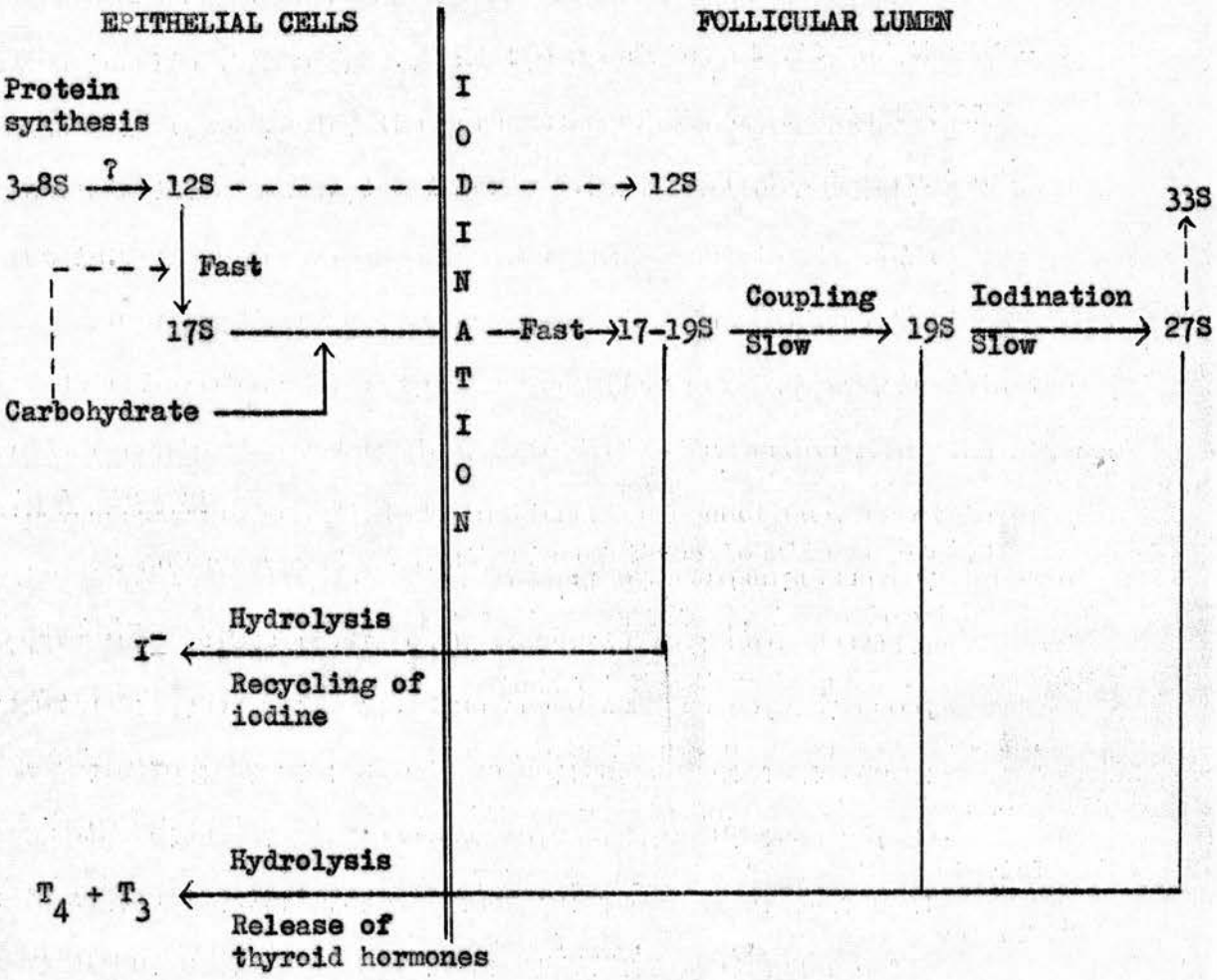
during centrifugation. It is only this minority of protein which would, by the above argument, contain ^{131}I-iodo-^{14}C-tyrosyl residues.

By calculation from the specific activity of ^{14}C-tyrosine-thyroglobulin (230 mμC/mg.) and the specific activity of the ^{14}C-amino acid (330μC/mole) the percentage of thyroglobulin totally labelled with ^{14}C-tyrosine is only 0.35% of the total amount of protein.

Final picture

It now appears that synthesis of thyroglobulin sub-units (possibly 3-8S, almost certainly 12S proteins), is followed by movement of these toward the apices of the thyroid cells. Probably at this stage the carbohydrate moiety is attached to the protein. It is not clear if the sub-units aggregate to form the 17S non-iodinated protein species at this stage. Iodinated 12S material has been demonstrated (Salvatore et al., 1965), but the level of iodination is less than 10% of the thyroglobulin level. It is possible that this is an artifactual dissociation occurring during isolation of the protein contaminating the uniodinated 12S material with sub-units from already iodinated thyroglobulin. The 12S component has been detected in only two mammals and it is probable that in the remainder, including sheep, aggregation to 17S protein occurs soon after release of the peptides from the rough endoplasmic reticulum. The non-iodinated protein probably passes from the apices of the epithelial cells and iodination commences at this stage or just after it has entered the lumen. The iodination proceeds to the monoiodination stage in the case of some tyrosyl residues and to diiodination in only a few cases. The specificity of this

iodination is undoubtedly influenced by the conformation of the protein and this, in turn, is altered as the iodination level increases. This process results in protein molecules exhibiting a range of properties, namely, an increase in sedimentation constant from 17S to 19S, corresponding both to increased molecular weight due to iodine and to a probable 'tightening' of the tertiary structure of the molecule. This 'maturation' stage is accompanied by coupling of a small number of diiodotyrosyl residues which may further increase the 'tightening' of the molecule. Interestingly, the aggregation of sub-units and further iodination is repeated in the formation of 27S protein probably from two 19S units. The 27S material contains up to 50% more iodine on a dry weight basis than does thyroglobulin. The 33S protein species may represent further aggregation of this type, although it has been suggested that this may occur artifactually during storage at low temperatures after freeze-drying. The proportion of 27S species is higher in protein from thyroids containing larger follicles which are less active, and, after labelling with $^{131}I_2$ the increase of this in 27S is much slower than that in 19S protein. This points to the further aggregation of 19S protein being a slow process. This again agrees with the theory that the maturation of 19S protein in the periphery of the lumen is slow and may occur only after the iodinated protein has diffused from the periphery where pincytosis occurs. This process is summarised overleaf:



- - - - - → Possible reaction step

——?——→ Doubtful step

If pinocytosis is the mechanism by which the epithelial cells ingest colloid, this will not differentiate between mature and immature protein. (Both species will be hydrolysed, with recycling of iodine via the deiodinases, and with release of thyroid hormone to the cell and then to the circulation.) This is not incompatible with the 'two pools of iodoprotein' suggested by Plaskett et al. (1963,a) as an explanation of the heterogeneity of the iodotyrosine fraction since this requirement is met by the fast and slow turnover of the proteins at the periphery and centre of the follicle respectively. Only after the protein has moved away from the area of most active reabsorption will it have time to mature fully and in some cases aggregate to larger units.

After pinocytosis both immature and mature protein will be hydrolysed. Iodide will be released from the iodotyrosines by the deiodinases and recycled. The thyroid hormones will be released only from the mature protein and will pass through the base of the epithelial cells into the blood.

APPENDIX I

Calculation of the sedimentation constants of the thyroglobulin species

The sedimentation constant of a protein undergoing ultracentrifugation is given by

$$S^{0\%}_{20^o,w} = \frac{d(\ln x)}{dt} \cdot \frac{1}{\omega^2} \cdot \frac{n_t}{n_{20^o}} \cdot \frac{1}{10^{-13}} \quad (1)$$

where $S^{0\%}_{20^o,w}$ is the sedimentation constant in Svedberg units, that is, 10^{-13} sec.$^{-1}$, corrected to zero protein concentration at 20° in water, where lnx is the natural logarithm of the distance in ~~metres~~ cms. of the protein peak from the centre of rotation at time t sec., where ω^2 is the angular velocity of the centrifuge rotor in radians per sec. and equals $\left(\frac{2\pi \text{ revolutions/min.}}{60}\right)^2$, and where n_t and n_{20^o} are the viscosities of water at temperatures t°C and 20°C.

Plates 1-4 (following p. 79) show the schlieren patterns of the moving boundaries of the protein species during ultracentrifugation. Two calibration holes cut in the balancing cell in the rotor appear as vertical dark bands to the left and right of the plates. The distance from the centre of rotation to the right hand edge of the inner, that is, right-hand, calibration hole was 5.70 cm. and the distance between the outer edges of the two holes was 1.60 cm. Using these measurements the changes in position of the four protein peaks on the plates were converted into actual distances moved from the centre of rotation.

The exact time of each photograph was found from the odometer reading halfway through the 10 sec. exposure. From the gear ratios

at 56,100 rev./min. one odometer reading was equivalent to 6.845 sec.

Calculation of the sedimentation constants of the fastest and slowest moving protein peaks

As these peaks could be identified on only two plates, the gradients, d(lnx)/dt, were taken from two pairs of points on the graph of lnx against time (Fig. 20, following p. 79).

During centrifugation the rotor temperature fell from 21.5° to 20.9°. The average temperature (21.2°) was used to correct for the decreased viscosity of water at 21.2° (0.976 centipoise) against that at 20.0° (1.005 centipoise).

Sedimentation constants decrease with increasing protein concentration and are corrected by extrapolation to zero protein concentration. The sedimentation constants of the major peak in 1.5%, 1.0% and 0.5% solutions of hog thyroglobulin are 16.0S, 17.1S and 18.2S, respectively (Malan, 1968). From these values the sedimentation constant at zero protein concentration was 19.3S. The correction factor for an 0.5% solution of sheep thyroglobulin was taken as 19,3/18.2, that is, 1.06.

Substitution in (1) and correction as detailed above gave sedimentation constants of 33.5S and 10.5S respectively for the heaviest and lightest proteins.

Calculation of the sedimentation constants for the two major peaks

The gradients, d(lnx)/dt, for the other two peaks were calculated by the method of linear regression.

For a line $q = bp + a$, the gradient b is given by

$$b = {}^{Cpq}/_{Cpp}$$

and the variance of the gradient is given by

$$Vb = \frac{1}{n-2}\left({}^{Cqq}/_{Cpp} - b^2 \right)$$

where q is the dependent variable, that is, lnx, and p is the independent variable, that is, time in sec., n is the number of determinations and

$$Cpp = \Sigma p^2 - (\Sigma p)^2/n,$$

$$Cqq = \Sigma q^2 - (\Sigma q)^2/n,$$

and $$Cpq = \Sigma pq - \Sigma p \Sigma q/n.$$

The gradients for the two peaks were found. After substitution of these in equation (1) and correction for the viscosity of water and for protein concentration the sedimentation constants were 19.15S and 27.16S. The variance and standard deviation for the gradient of the 19S peak were calculated giving the final value of the sedimentation constant as 19.15 ± 0.35S.

APPENDIX II

Calculation of the moles of peptide N_9 per mole of thyroglobulin by conversion of the monoiodotyrosine from N_9 to diiodotyrosine by $^{131}I_2$ of known specific activity

The quantities of monoiodotyrosine iodinated under the conditions outlined on p. 125 had been isolated initially from 0.914 nanomoles thyroglobulin.

Hydrolysis of the thyroglobulin with α-chymotrypsin was, in the light of the autotitration work, assumed to be 100%. Elution of the peptide from the peptide maps has been shown to be greater than 99.5% efficient. Pronase hydrolysis of N_9 released 73.6% of the monoiodotyrosine (Table 38, following p. 108). In this Table there appears to be 15% of diiodotyrosine in the peptide, but, after separation of the two components of N_9, both of these were, after complete pronase hydrolysis, shown to contain very nearly 100% monoiodotyrosine (Table 40, following p. 108).

Complete iodination was achieved with 160 nanomoles of $^{131}I_2$. One-fifth of the product was chromatographed in BA and the diiodotyrosine peak found to have ^{131}I-activity of 5,655 counts/min.: equivalent to 12.8 mμC with an efficiency of 20.0%. This activity had been corrected for the residual activity in the monoiodotyrosine and all activities corrected to the same day. The specific activity of the $^{131}I_2$ used for iodination was 37.8 μC/mg. Thus 12.8 mμC was equivalent to 12.8/37.8 μg = 0.338 μg to 1.33 nanomole.

The molecular weight of iodine was taken as 254 on the assumption that both the iodine atoms of the diiodotyrosine formed had equilibrated with the added $^{131}I_2$.

This quantity of diiodotyrosine was one-fifth of the sample and, after correction for the 73.6% release of monoiodotyrosine from N_9, was equivalent to 9.02 nanomoles.

This had been released from 0.914 nanomoles of thyroglobulin.

N_9 was known to be composed of two monoiodotyrosine-containing peptides comprising 64.1% and 35.9% of its activity. It is not possible to say if these activities are proportional to the quantities of the two peptides isolated or, as seems more reasonable, if the two peptides differ in their degrees of iodination.

Accepting the latter idea, each mole of thyroglobulin contains 9.02/0.914 = 9.87 moles of 'N_9', that is, approximately 5 moles of each of the component peptides.

Calculation of the moles of peptide A_5 and the moles of the uniodinated form of A_5 per mole of thyroglobulin by conversion of the monoiodotyrosyl residue to a diiodotyrosyl residue with $^{131}I_2$ of known specific activity

After A_5 and its associated tyrosine-containing peptide (T_5) had been separated by electrophoresis at pH 8.2 (p. 132), both peptides were iodinated and electrophoresed at pH 6.5 (Figs. 30, following p. 129, and 32, following p. 132).

The peptides were derived initially from 3.18 nanomoles of thyroglobulin and iodinated with 800 nanomoles of $^{131}I_2$ with a specific activity of 165 μC/mg. All ^{131}I-activities were corrected to the same date.

On this date the activity of A_5 was 79 counts/min. After iodination A_5 and T_5 had activities of 1,935 and 1,097 counts/min. (for identification of the products of iodination see pp. 129-132), equivalent to 4.18 mμC and 2.47 mμC or 7.48 nanomoles and 4.40 nanomoles respectively.

There were 2.35 moles of A_5 and 1.38 moles of T_5 per each mole of thyroglobulin. On the assumption that these are the iodinated and uniodinated forms of one peptide, this is present as 3.73, or nearly 4, moles per mole of thyroglobulin.

Thyroglobulin is known to be formed from, and to dissociate into, sub-units with sedimentation constants of 12S. Further dissociation has been achieved by Edelhoch (1965) to give a globular form (M) with a molecular weight approximately half that of the 12S species. This was achieved by mild reduction and this M form appears to be the limiting size obtainable in the absence of peptide bond rupture, since the molecular weight observed in 5M guanidine, after complete reduction, is not less than that of the M form.

Only 3-4 N-terminal amino acid residues have been found in thyroglobulin (Dopheide and Trikojus, 1964).

From this data thyroglobulin appears to comprise four sub-units of approximately 165,000 molecular weight. This agrees reasonably with the iodination results of 5,5 and [illegible] moles of peptide per mole of thyroglobulin.

Table 47. Amino acid composition of a basic peptide from PMI(^{14}C-Prot.Hyd.)Ch8/4

Acid hydrolysis of the peptide was followed by separation of the amino acids by a modified peptide-mapping technique

Amino acid	^{14}C-activity (counts/min.)	No. of C-atoms	Relative* specific activity of C-atoms	Relative+ activities (counts/min.)	Mole ratios of amino acid
Phenyl-alanine	560	9	0.460	135	1.00
Leucine/ Isoleucine	830	6	0.463	295	2.18
Arginine	950	6	0.412	384	2.94
Lysine	380	6	0.338	187	1.38

* Data from Table 33

\+ Reduction of the ^{14}C-activities to a molar basis

APPENDIX III

Determination of the total amino acid composition of a peptide

A method has been outlined on p. 84 whereby the amino acid composition of a peptide, in very small quantity, can be determined. This method depends on the total acid hydrolysis of the isolated peptide, separation of the released amino acids (p. 84) and detection of these by their ^{14}C-activities.

A basic peptide, well separated from its neighbours, (see Plate 8, following p. 99, the autoradiograph of the ^{14}C-activity of PMI(^{14}C-Prot.Hyd.)Ch.8/4 where the peptide is arrowed) was selected. This peptide was eluted, taken to dryness, dissolved in 5.7N HCl, sealed in a glass capillary and heated at 105° for 21 hr. After removal of excess HCl by alternate rotary evaporation and solution in distilled water, the amino acids were separated by the modified peptide mapping technique (p. 84).

Scanning the paper after electrophoresis revealed ^{14}C-activity only among the basic and neutral amino acids. After chromatography in BA arginine and lysine separated. The neutral material yielded two radioactive spots with R_f values corresponding to phenylalanine and leucine or isoleucine.

The ^{14}C-activities of the four radioactive areas were found (Table 47) and were divided by the number of carbon atoms in each to bring the activities to a molar basis.

At this stage, two assumptions were made. First, that all the residues of a particular amino acid in thyroglobulin became equally labelled, and, secondly, that the specific activities of all the amino acid residues were the same.

The first point could not be shown directly. It is certainly true that in very short incubations the exogenous ^{14}C-amino acids add on to partially finished peptide chains in such a way that the C-terminal end can be shown to be much more highly labelled than the N-terminal end of the chain. In this case, however, the incubation had continued for 8 hr. and in Fig. 7(following p. 53) it can be seen that the intracellular soluble protein is most highly labelled after only 2 hr. The ^{14}C-amino acids used for the labelling came from universally labelled Chlorella protein hydrolysate.

The second assumption was not valid, as it had already been shown that a certain amount of interconversion between amino acids occurs before these are incorporated into protein. These changes would be expected to increase over the period of incubation and be reflected in changes in the specific activities of some of the amino acids.

This complication was avoided because the thyroglobulin, from which the original peptide came, had already been hydrolysed and the specific activity of each amino acid found (Section III.7, and Table 33, following p. 84).

Division of the activities of the ^{14}C-atoms in the amino acids from the peptide by their respective specific activities reduced the activities to a relative molar basis (Table 47, column 4). These were then directly comparable and division by the smallest revealed the lowest multiple of the molar ratio for the amino acids (Table 47, column 5). The formula of the peptide is postulated as $(Lys,(Arg)_3,(Leu/Ileu)_2,Phe.OH)_n$ where phenylalanine is most probably the C-terminal amino acid following chymotryptic digestion.

As most peptides were of small molecular size, n probably equals 1, giving a molecular weight of 947.

MATERIALS

Reagents	Supplier
All common chemicals were 'Analar' grade reagents. ATP, GTP	British Drug Houses, Poole, U.K.
Thiouracils, ascorbic acid, creatine phosphate, creatine phosphokinase	Koch-Light Labs. Ltd., Colnbrook, U.K.
Carrier-free $Na^{131}I$ or $Na^{125}I$ solutions and ^{14}C-amino acids	The Radiochemical Centre, Amersham, Bucks.
'Sephadex'	Pharmacia, Uppsala, Sweden.
'Ilfex' No-screen, envelope-packed X-ray films	Ilford Ltd., Ilford, U.K.

A direct-reading pH meter (Electronic Instruments Ltd.) was used with an 'all purpose' glass electrode (Type GHS 23), a calomel electrode and a resistance thermometer.

The source of enzymes and a description of other equipment used are given in the text.

REFERENCES

Acher, R. & Crocker, C. (1952). Biochem. Biophys. Acta 9, 704

Alexander, N.M. (1965). Current Topics in Thyroid Research, 43. Ed. Cassano C. & Andreoli M. Academic Press Inc.

Allen, E.H. & Schweet, R.S. (1962). J. Biol. Chem. 237, 760

Andrews, P. (1964). Biochem. J. 91, 222

Barzelatto, J., Murray, I.P.C. & Stanbury, J.B. (1962). Endocrinol. 70, 328

Benabdeljlil, C., Michel-Bechet, M. & Lissitsky, S. (1967). Biochem. Biophys. Res. Commun. 27, 74

Blum, F. (1927). Schweiz Med. Wochschr. 57, 808

Blum, F. & Grützner, R. (1914). Z. Physiol. Chem. 85, 429

Bois, I. & Larsson, L.C. (1958). Acta Endocrinol. Kbh. 28, 262

Boyd, G.S. & Oliver, M.F. (1960). J. Endocrinol. 21, 25

Carnegie, P.R. (1965). Biochem. J. 95, 9P

Campbell, P.N., Greengard, O. & Kernot, B.A. (1960). Biochem. J. 74, 107

De Groot, L.J. & Davis, A.M. (1961,a). J. Biol. Chem. 236, 2,009

De Groot, L.J. & Davis, A.M. (1961,b). Endocrinol. 69, 695

De Groot, L.J. & Davis, A.M. (1962). Endocrinol. 70, 492

Derrien, Y., Michel, R., Pedersen, K.O. & Roche, J. (1949). Biochem. Biophys. Acta, 3, 436

Derrien, Y., Michel, R. & Roche, J. (1948). Biochem. Biophys. Acta. 2, 454

Dixon, M. & Webb, E.C. (1961). Adv. Prot. Chem. 16, 197

Dixon, M. & Webb, E.C. (1964). Enzymes. 2nd ed. Longmans

Dopheide, T.A.A. & Trikojus, V.M. (1964). Nature 201, 1,128

Dulbecco, R. & Vogt, M. (1954). J. Expt. Med. 99, 167

Edelhoch, H. (1960), J. Biol. Chem. 235, 1,326

Edelhoch, H. (1962). J. Biol. Chem. 237, 2,778

Edelhoch, H. & Perlman, R.L. (1968). J. Biol. Chem. 243, 2,008

Edholm, J. (1961). J. Ultrastruct. Res. 5, 575

Falconer, I.R. (1967). Mem. Soc. Endocrinol. 15, 141

Fink, K. & Fink, R.M. (1948). Science, 108, 358

Freinkel, N. & Ingbar, S.H. (1955). J. Clin. Endocrinol. Metab. 15, 598

Fromageot, C., Jutisz, M., Lafon, M. & Roche, J. (1948). Compte Rendu Soc. Biol. 142, 785

Gross, J. & Pitt-Rivers, R. (1952). Lancet 262, 439

Gross, J. & Leblond, C.P. (1951). Endocrinol. 48, 714

Haddad, H.M. & Sidbury, J.B. (1959). J. Clin. Endocrinol. Metab. 19, 1,446

Halder, D., Freeman, K. & Work, T.S. (1966). Nature 211, 9

Halmi, N.S. & Pitt-Rivers, R. (1962). Endocrinol. 70, 660

Harington, C.R. (1926,a). Biochem. J. 20, 293

Harington, C.R. (1926,b). Biochem. J. 20, 300

Harington, C.R. & Barger, G. (1927). Biochem. J. 21, 169

Harington, C.R. & Pitt-Rivers, R. (1945). Biochem. J. 39, 157

Harington, C.R. & Randall, S.S. (1929). Biochem. J. 23, 373

Hoagland, M.B. (1960). Nucleic Acids. Vol. III. 351. Ed. Chargaff, E. & Davidson, J.N. Academic Press Inc.

Hoagland, M.B., Keller, E.B. & Zamecnik, P.C. (1956). J. Biol. Chem. 218, 345

Hultin, T., Leon, H. A. & Cerasi, E. (1961). Expt. Cell. Res. 25, 345

Johnson, T.B. & Tewkesbury, L.B. (1942). Proc. Nat. Acad. Sci. Wash., 28, 73

Keller, E.B. & Zamecnik, P.C. (1956). J. Biol. Chem. 221, 45

Kendall, E.C. (1915). J. Amer. Med. Ass. 64, 2,042

Korner, A. (1962). Biochem. J. 83, 69

Lamfrom, H. (1961). J. Mol. Biol. 3, 241

Levenson, V.J. (1961). Biull. eksp. Biol. Med. 49, 62

Li, C.H. (1945). J. Amer. Chem. Soc. 67, 1,065

Lissitsky, S. (1966). Acta Endocrinol. 53, 292

Littlefield, J.W. & Keller, E.B. (1957). J. Biol. Chem. 224, 13

Ludwig, W. & Mutzenbecher, P. von (1939). Z. Physiol. Chem. 258, 195

McGirr, E.M., Hutchison, J.H. & Clement, W.E. (1959). Lancet 1959-II, 823

Malan, P.G. (1968). Ph.D. Thesis, University of Edinburgh

Maloof, F., Sato, G. & Soodak, M. (1964). Medicine 43, 375

Mann, W., Leblond, C.P. & Warren, S.L. (1942). J. Biol. Chem. 142, 905

Marine, D. & Rogoff, J.M. (1916). J. Pharmacol. 8, 439

Martin, R.G. & Ames, B.N. (1961). J. Biol. Chem. 236, 1,372

Mauchamp, J., Macchia, V. & Nunez, J. (1965). Current Topics in Thyroid Research, 172. Ed. Cassano, C. & Andreoli, M. Academic Press Inc.

Mayberry, W.E., Rall, J.E. & Berman, M. (1965). Current Topics in Thyroid Research, 61. Ed. Cassano, C. & Andreoli, M. Academic Press Inc.

Michl, H. (1951). Monatsch. Chem. 82, 489

Morais, R. & Goldberg, I.H. (1967). Biochemistry, 6, 2,538

Morton, M.E. & Chaikoff, I.L. (1943). J. Biol. Chem. 147, 1

Murray, I.P.C. & McGirr, E.M. (1964). The Thyroid Gland, Vol. 2, 39. Ed. Pitt-Rivers, R. & Trotter, W.R. Butterworths.

Nadler, N.J. (1965). Current Topics in Thyroid Research, 73. Ed. Cassano, C. & Andreoli, M. Academic Press Inc.

Nunez, J., Mauchamp, J. Macchia, V., Jerusalami, A. & Roche, J. (1965). Biochem. Biophys. Res. Commun. 20, 71

Nunez, J. Mauchamp, J. & Roche, J. (1964). Biochem. Biophys. Acta 86, 361

Orr, J.B. & Leitch, I. (1929). Iodine in Nutrition. Special Report Series of the Medical Research Council, No. 123

Oswald, A. (1909). Arch. Exp. Path. Pharmak. 60, 115

Perlman, I., Chaikoff, I.L. & Morton, M.E. (1941). J. Biol. Chem. 139, 433

Pitt-Rivers, R. (1948). Biochem. J. 43, 223

Pitt-Rivers, R. (1962). Biochem. J. 82, 108

Pitt-Rivers, R. & James, A.T. (1958). Biochem. J. 70, 173

Pitt-Rivers, R. & Tata, J.R. (1959). Ed. The Thyroid Hormones, Int. Ser. of Mon. on Pure and App. Biol.

Plaskett, L.G. (1961). Biochem. J. 78, 657

Plaskett, L.G. (1964). Chromatographic Reviews, 91. Ed. Lederer, M. Elsevier.

Plaskett, L.G., Barnaby, C.F. & Lloyd, G.I. (1963,a). Biochem. J. 87, 473

Plaskett, L. G., Barnaby, C.F. & Lloyd, G.I. (1963,b). Biochem. J. 89, 479

Poulik, M.D. & Smithies, O. (1958). Biochem. J. 68, 637

Pulvertaft, R.J.V., Davies, J.R., Weiss, L. & Wilkinson, S.H. (1959). J. Path. Bact. 77, 19

Querido, A., Stanbury, J.B., Kassenar, A.A.H. & Meijer, J.W.A. (1956). J. Clin. Endocrinol. Metab. 16, 1,096

Ragupathy, E., Abraham, S., Kerkof, P.R. & Chaikoff, I.L. (1964). Endocrinol. 74, 468

Robertis, E. de (1941). Anat. Record 80, 219

Roche, J., Lissitsky, S. & Michel, R. (1952,a). Compte Rendu Acad. Sci. Paris. 234, 997

Roche, J.,Lissitsky, S. & Michel, R. (1952,b). Compte Rendu Acad. Sci. Paris. 234, 1,228

Roche, J., Michel, O., Michel, R., Gorbman, A. & Lissitsky, S. (1953). Biochem. Biophys. Acta 12, 570

Roche, J. & Michel, R. (1951). Adv. Prot. Chem. 6, 253

Roche, J., Michel, R. & Lafon, M. (1947). Biochem. Biophys. Acta 1, 453

Roche, J., Michel, R., Michel, O., & Lissitsky, S. (1952). Biochem. Biophys. Acta 9, 161

Salvatore, G., Sena, L., Viscidi, E. & Salvatore, M. (1965). Current Topics in Thyroid Research, 193. Ed. Cassano, C., & Andreoli, M. Academic Press Inc.

Salvatore, G., Salvatore, M., Cahnmann, H.J. & Robbins, J. (1964). J. Biol. Chem. 239, 3,267

Seed, R.W. & Goldberg, H. (1963). Proc. Nat. Acad. Sci. U.S. 50, 275

Sela, M. & Sarid, S. (1956). Nature 178, 540

Shulman, S. & Armenia, J.P. (1963). J. Biol. Chem. 238, 2,723

Shulman, S., Rose, N.R. & Witebsky, E. (1955). J. Immunol. 75, 291

Simon, C., & Croz, B. (1965). Current Topics in Thyroid Research, 77 Ed. Cassano, C. & Andreoli, M. Academic Press Inc.

Smith, I. (1960). Chromatographic and Electrophoretic Techniques, Vol. II

Smithies, O. (1955). Biochem. J. 61, 629

Soffer, R.L. (1967). J. Mol. Biol. 10, 359

Soffer, R.L. & Mendelsohn, N. (1966). Biochem. Biophys. Res. Commun. 23, 252

Spiro, R.G. & Spiro, M.J. (1965). Current Topics in Thyroid Research, 157. Ed. Cassano, C. & Andreoli, M. Academic Press Inc.

Stanbury, J.B. (1960). Proc. Roy. Soc. Med. 53, 904

Stanbury, J.B. (1967). Mem. Soc. Endocrinol. 15, 107

Stanbury, J.B. & Hedge, A.N. (1950). J. Clin. Endocrinol. 10, 1,471

Stanbury, J.B., Ohela, K. & Pitt-Rivers, R. (1955). J. Clin. Endocrinol. 15, 54

Stolc, V. & Langer, P. (1963). Physiol. Bohemoslovenica 12, 251

Synge, R.L.M. & Youngson, M.A. (1961). Biochem. J. 78, 31P

Tang, J. (1963). Nature 199, 1,094

Tata, J.R. (1958). Biochem. J. 72, 222

Tong, W. & Chaikoff, I.L. (1958). J. Biol. Chem. 232, 939

Tong, W., Kerkof, P. & Chaikoff, I.L. (1962). Biochem. Biophys. Acta 60, 1

Tong, W., Taurog, A. & Chaikoff, I.L. (1957). J. Biol. Chem. 227, 773

Ui, N. & Tarutani, O. (1961). J. Biochem. (Tokyo). 50, 508

Umbreit, W.W., Burris, R.H. & Stauffer, J.F. (1964). Ed. Manometric Techniques (4th ed.)

van Zyl, A. & Edelhoch, H. (1967). J. Biol. Chem. 242, 2,423

Weiss, B. (1953). J. Biol. Chem. 205, 193

Wolff, J. & Chaikoff, I.L. (1947). Endocrinol. 41, 295

Wollman, S.H. (1965). Current Topics in Thyroid Research, 1. Ed. Cassano, C. & Andreoli, M. Academic Press Inc.

Yamazaki, I. (1958). Proc. Intern. Symposium on Enzyme Chem., Tokyo and Kyoto, 224-9

Yip, C.C. (1965). Biochem. Biophys. Acta 96, 75

Zamecnik, P.C. & Keller, E.B. (1954). J. Biol. Chem. 209, 337

Zamecnik, P.C. & Keller, E.B. (1957). J. Biol. Chem. 224, 24

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